The role of metabotropic glutamate and NMDA receptors in hippocampal long-term potentiation and spatial learning

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In accordance with the University of Edinburgh postgraduate study regulation 3.8.7, I declare that this thesis was composed by myself, and the work presented herein is my own except where otherwise indicated.

S. J. Martin

To my parents

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Abstract

The experimental phenomenon of long-term potentiation (LTP) is widely considered to provide a model of the cellular mechanisms underlying memory formation. NMDA receptor-dependent LTP is readily induced in a number of pathways within the hippocampal formation, a brain structure known to be involved in various forms of learning. It is believed that the cellular mechanisms by which memories are formed within the hippocampus are similar, or identical, to the cellular mechanisms of LTP. Indeed, treatments that prevent the induction of LTP, such as the blockade of NMDA receptors, also prevent certain forms of spatial learning.

The aim of the present thesis was firstly to investigate whether the behavioural deficit resulting from NMDA receptor blockade reflects a genuine learning impairment, rather than a disturbance of some other aspect of brain functioning. The behavioural test used was a reference memory task in the watermaze, the learning of which is highly sensitive to hippocampal dysfunction. It has been claimed that the apparent learning deficits caused by the application of NMDA receptor antagonists may be secondary to sensorimotor disturbances, or drug-induced brain damage. Behavioural and histological analysis in the present study was not consistent with either of these two possibilities. However, owing to the fact that NMDA receptor blockade does indeed cause a number of side effects, subsequent experiments investigated the role of a novel class of receptor, the metabotropic glutamate receptor (mGluR), in both LTP and spatial learning. Previous studies have suggested that mGluR activation is necessary for both LTP induction and the acquisition of spatial memories. However, in the studies described herein, a broad-spectrum mGluR antagonist, MCPG, caused a modest impairment in spatial learning, but only under certain circumstances. Moreover, MCPG had no effect on the induction of LTP *in vivo*, despite the fact that the application of MCPG successfully blocked the increase in spontaneous hippocampal activity induced by the application of an mGluR agonist, ACPD. The role of mGluRs in hippocampal functioning is discussed in the light of these findings.

Finally, electrophysiological experiments were conducted in order to investigate the activity-dependent reversal of LTP. It was found that the delivery of low frequency stimulation within 2 min of LTP induction caused a total reversal of LTP, but had little effect on baseline EPSPs when delivered alone. The ability to selectively erase recently induced potentiation in this way might provide an alternative to pharmacological intervention in future studies of the role of LTP-like mechanisms in memory storage.

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LIST OF ABBREVIATIONS

ACPD	1-aminocyclopentane-1,3-dicarboxylic acid
aCSF	artificial cerebrospinal fluid
t-ADA	*trans*-acetidine-2,4-dicarboxylic acid
AIDA	(*R*,*S*)-1-aminoindan-1,5-dicarboxylic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP	anteroposterior
AP3	amino-3-phosphonopropionate
AP4	2-amino-4-phosphonobutyrate
AP5	2-amino-5-phosphopentanoate
AP7	2-amino-7-phosphoheptanoate
4-AP	4-aminopyridine
APDC	(2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylic acid
APP	β-amyloid precursor protein
ATP	adenosine triphosphate
CA	cornu ammonis
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CAN	calcium-activated non-specific cation current
4C3HPG	4-carboxy-3-hydroxyphenylglycine
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
4CPG	4-carboxyphenylglycine
CPP	3-((-)-2-carboxypiperazin-4-yl) propyl-1-phosphonate
CPPG	(*R*,*S*)-α-cyclopropyl-4-phosphono-phenylglycine
CREB	calcium response element binding protein
CS	conditioned stimulus
DAG	diacylglycerol
DCG-IV	(2*S*,1'*R*,2'*R*,3'*R*)-2-(2,3-dicarboxycyclopropyl)glycine
DHPG	dihydroxyphenylglycine
DNMS	delayed non-matching-to-sample
DRL	differential reinforcement of low rates
DV	dorsoventral
EEG	electroencephalogram

EGLU	(2*S*)-α-ethylglutamate
EGTA	ethylene-bis (oxyethylenenitrate) tetra-acetic acid
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
E-S	EPSP / spike
fEPSP	field excitatory post synaptic potential
GABA	γ-aminobutyric acid
H + E	haematoxylin and eosin
3-HPG	3-hydroxyphenylglycine
HPLC	high performance liquid chromatography
HSP72	heat shock protein 72
I / O	input / output
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
I.Q.	intelligence quotient
I_{AHP}	afterhyperpolarization current
$I_{K(slow)}$	slowly inactivating voltage-dependent potassium conductance
I_M	slow, non-inactivating voltage-dependent potassium conductance
IP3	inositol trisphosphate
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
ITI	inter-trial interval
l.f.s.	low frequency stimulation
Lat.	lateral
L-CCG-I	(2*S*,1'*S*,2'*S*)-2-(carboxycyclopropyl)glycine
L-CSA	L-cysteine sulphinate
LGN	lateral geniculate nucleus
L-LTP	late LTP
L-SOP	L-serine-O-phosphate
LTD	long-term depression
LTP	long-term potentiation
MAP4	α-methyl-4-phoshonobutyrate
MAPK	mitogen activated kinase
MCCG	α-methyl-CCG-I
MCPG	α-methyl-4-carboxyphenylglycine
mEPSC	miniature excitatory postsynaptic potential
mGluR	metabotropic glutamate receptor

MK-801	(+)-5-methyl-10,11-dihydro-5h-dibenzo [a,d] cyclohepten-5,10-imine (dizocilpine)
MPPG	α-methyl-4-phosphonophenylglycine
mRNA	messenger ribonucleic acid
MSOP	(*R*,*S*)-α-methylserine-O-phosphate
MSOPPE	(*R*,*S*)-α-methylserine-O-phosphate monophenyl-phosphoryl ester
MSPG	α-methyl-4-sulphonophenylglycine
MTPG	α-methyl-4-tetrazoylphenylglycine
NMDA	*N*-methyl-D-aspartate
NS	not significant
PBS	phosphate buffered saline
PCCG-IV	(2*S*,1'*S*,2'*S*,3'*R*)-2-(2'-carboxy-3'-phenylcyclopropyl)glycine
PCP	phencyclidine
PKA	protein kinase A
PKC	$Ca^{2+/}$phospholipid-dependent protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PPF	paired-pulse facilitation
PTP	post-tetanic potentiation
REM	rapid eye movement
STP	short-term potentiation
TT	transfer test
US	unconditioned stimulus
VSCC	voltage-sensitive calcium channel

Chapter One

Thesis overview

1.1 Memory: a role for hippocampal synaptic plasticity?

An understanding of the physiological basis of memory remains one of the central goals of contemporary neuroscience. Many definitions of memory have been proposed, and many different types of memory have been identified. However, most forms of memory are characterized by a change in behaviour as a result of prior experience. The process leading to such a change is known as learning. In order to result in such a behavioural change, mammalian learning must result in a modification of the central nervous system. The nature and location of this modification has been the focus of a vast amount of research, much of it centred around a forebrain structure called the hippocampus.

The hippocampus has attracted particular interest for a number of different reasons. For instance, hippocampal damage in humans can result in a severe deficit in the formation of memories for facts and events, an impairment that corresponds well to the everyday notion of a "bad memory". Furthermore, hippocampal lesions in rodents have suggested that the hippocampus may be particularly involved in the formation of internal representations of space, or "cognitive maps" (see O'Keefe and Nadel, 1978). Compared to many other postulated high level cognitive processes, the suggestion that a structure is involved in the processing of "space" generates a number of experimentally testable predictions, making the hippocampus an attractive brain region for experimental psychological research.

In addition, the hippocampus has an apparently rather simple and well-defined anatomical structure. The principal intrahippocampal connections have been known for some time, and a computational analysis of the "algorithm" performed by the hippocampal circuitry may ultimately prove possible. Although such a "circuit level" analysis of hippocampal function is currently beyond our reach, the discovery of a phenomenon known as long term potentiation (LTP) has provided a huge impetus to the field of hippocampal research. LTP is an experimentally induced form of synaptic plasticity in which certain hippocampal glutamatergic synapses exhibit a lasting increase in the efficacy with which they transmit afferent signals. Such changes in synaptic strength had previously been postulated on purely theoretical grounds, and the phenomenon displays many of the properties desirable in a cellular mechanism of associative learning (see chapter 2.4).

Since the discovery of hippocampal LTP over 30 years ago, a considerable experimental effort has been devoted to the attempt to link memory formation with LTP-like phenomena in the hippocampus. Indeed, it is widely assumed on *a priori* grounds that changes in synaptic efficacy must underlie certain forms of learning. The demonstration that networks of "neuron-like" elements with modifiable connections between them can exhibit certain forms of learning has added to this view (Gluck and Rumelhart, 1990). Furthermore, it has been demonstrated that certain simple forms of

associative learning in invertebrates such as *Aplysia*, are dependent on the modification of synaptic strength between pairs of connected neurons (Carew et al., 1981). However, the evidence suggesting that synaptic plasticity underlies learning in the mammalian brain, although extensive, is insufficient to prove such a link definitively.

One conceptual issue concerns the fact that mammalian LTP is an experimental phenomenon, involving an increase in synaptic efficacy induced by the massive synchronous high frequency activation of a large population of afferent fibres. Such patterns of activity do not occur naturally, although limited periods of high frequency bursting are likely to occur in small distributed populations of afferents. Hence, the hypothesis that LTP is the basis of learning means simply that the cellular mechanisms artificially engaged during the induction of LTP are the same as those engaged during learning. LTP cannot, of course, be literally equated with learning, and the LTP / learning hypothesis should be understood only in the more limited sense proposed.

1.2 Introduction to the experimental work

The experiments described throughout this thesis constitute an attempt to address the LTP / learning hypothesis as outlined above. In some instances, the mechanistic equivalence of LTP and learning has been investigated by comparing the effects of a treatment on LTP with its effects in a learning task. However, in many cases, purely electrophysiological experiments have been carried out in an attempt to identify ways of modulating LTP that might, if successful, generate alternative approaches to the investigation of the LTP / learning relationship.

1.2.1 Does pharmacological blockade of hippocampal NMDA receptors result in a genuine learning impairment?

Probably the most common approach for testing the LTP / learning hypothesis has been to investigate the effects of a pharmacological treatment on both long-term potentiation and learning. For instance, the blockade of hippocampal NMDA receptors impairs learning at a dose equivalent to the dose at which it blocks LTP (Davis et al., 1992). However, it has been suggested that NMDA receptor antagonists impair behaviour by some mechanism unrelated to their effects on LTP. For instance, the administration of the commonly used NMDA antagonist AP5 causes sensorimotor disturbances and has also been suggested to cause brain damage (e.g. Cain et al., 1996).

The first experiments of this thesis, described in chapter 5, represent an attempt to discover whether the impairment induced by intraventricular infusion of AP5 is consistent with either of the above possibilities. Rats were tested using a watermaze reference memory protocol similar to that described by Cain and colleagues. Training was carried out either during AP5 infusion in order to investigate sensorimotor impairments, or after a period of chronic AP5 infusion in order to assess any residual deficit attributable to brain damage. A detailed histological analysis was carried out in order to further investigate the possibility of AP5-induced neurotoxicity.

The results of this study provide evidence to refute some of the arguments for a non-mnemonic impairment following treatment with an NMDA receptor antagonist. However, the fact that such drugs do undeniably cause sensorimotor impairments is undesirable. Hence, the remainder of the thesis represents an attempt to find an alternative way of modulating LTP without the drawbacks associated with the administration of NMDA receptor antagonists.

1.2.2 Does pharmacological blockade of hippocampal metabotropic glutamate receptors block hippocampal LTP and spatial learning?

The metabotropic glutamate receptor provides an attractive alternative target for pharmacological treatment. Blockade of this class of receptor has been reported either to limit the induction of LTP *in vivo* to approximately 2 h, or to block LTP completely, depending on the concentration of antagonist applied (Riedel et al., 1995a). This result might be exploited in order to probe the relationship between LTP and learning. Chapter 6 describes a series of experiments designed to characterize the effects of MCPG on performance in a simple reference memory task. The effects of MCPG on hippocampal LTP are investigated in chapter 7.

1.2.3 Are there alternatives to pharmacological intervention?

So far, all the experiments described have investigated the effects of the pharmacological antagonism of receptors believed to be involved in the induction of LTP. However, this approach has a number of limitations. For instance, no method of drug delivery is ideal, and some may result in substantial drug concentrations in structures surrounding the hippocampus. Secondly, particular receptors may indeed be involved in both LTP and learning, but the underling mechanisms may be different in each case.

In an attempt to overcome these problems, I decided to investigate an electrophysiological phenomenon that might offer the possibility of reversing LTP in potentiated synapses only. Pilot studies indicated that dentate LTP *in vivo* could only be reversed by low-frequency stimulation delivered very soon after tetanization, and a similar result had previously been reported in area CA1

in vitro (Stäubli and Chun, 1996). In order to characterize this phenomenon more fully, a detailed investigation of the time-dependence of LTP reversal was carried out in the dentate gyrus *in vivo* (see chapter 8). The time-dependent reversal of LTP might provide a tool for selectively targeting recently potentiated synapses after behavioural training, and hence investigating the role of LTP in memory retention, rather than acquisition. Such a study would be possible, although technically very demanding for reasons discussed in chapter 8.5.4. Pilot studies have not yet been carried out.

1.2.4 LTP induction: methodological issues

A final methodological chapter is devoted to issues associated with the successful induction of LTP, or its failure (chapter 9). Throughout the period during which the work described in this thesis was carried out, intermittent problems were experienced with the induction of lasting LTP. A number of possible factors were considered, including the initial size of the EPSP, the tetanus intensity used, the baseline stimulation intensity, the strain of rat, the effects of stress, and the possible influence of seasonal cycles. A number of these factors, including baseline stimulation parameters were critically important in determining the level of LTP recorded, a potentially worrying finding considering the arbitrary nature of the baseline parameters used by most investigators, including myself. The implications of these findings for future studies of LTP are discussed.

Chapter Two

Hippocampal synaptic plasticity and spatial learning

2.1 Introduction

The aim of this chapter is to provide a broad introduction to the hippocampus, with particular emphasis on the functions and mechanisms of hippocampal synaptic plasticity. Owing to the vast scope of this chapter, which could include topics ranging from molecular neurobiology to psychology, the material presented is necessarily selective. However, an attempt has been made to provide an integrated view of both the physiological and behavioral levels of analysis, a bridging of levels that is necessary if the potential role of synaptic plasticity in learning is to be understood.

The first few sections provide a general introduction to the anatomy of the hippocampus and its role in memory, both in humans and in animals. Following this, the mechanisms and possible significance of various forms of hippocampal synaptic plasticity are discussed. The final section represents an attempt to draw together several lines of evidence linking rodent hippocampal synaptic plasticity with learning. The literature review is by no means exhaustive, but is intended to provide an indication of the current state of the LTP / learning hypothesis, as well as placing my own experimental work within the context of existing studies.

2.2 Hippocampal Anatomy

2.2.1 Introduction

The mammalian hippocampal formation is located within the forebrain and forms part of the temporal lobe of the cerebral cortex. It is an elongated structure that lies beneath the corpus callosum and curves caudally away from the septum, and latero-ventrally around the thalamus. The two hippocampi are joined at their anterior point by the hippocampal commisure. The hippocampal formation (often simply referred to as the "hippocampus") is generally considered to consist of the hippocampus proper, i.e. Ammon's horn (Cornu Ammonis, CA), the dentate gyrus (fascia dentata), the subicular complex, and the entorhinal cortex (see figure 2.2A). Unlike the 6-layered structure of the neocortex, the dentate gyrus and Ammon's horn are 3-layered allocortical structures, with one principal cell type.

2.2.2 Subfields of the hippocampus proper

The hippocampus proper is divided into three subfields on the basis of cell size and distribution, regions CA1, CA2, and CA3, according to the terminology of Lorente de Nó (1934). Pyramidal cells in area CA3 receive a prominent mossy fibre input from the dentate gyrus, a projection absent in CA1. The CA2 region is less clearly defined, and its very existence has sometimes been debated.

A similar laminar organization is seen in all layers of the hippocampus proper. The pyramidal cell layer is most prominent, and deep to this layer is a relatively cell-free region called the stratum oriens. Above the pyramidal cell layer lies the stratum radiatum in which CA3 to CA1 Schaffer collateral connections and CA3 associational connections are located. The most superficial layer is known as the stratum lacunosum-moleculare, in which perforant path fibres from the entorhinal cortex terminate.

2.2.3 The dentate gyrus

Like the hippocampus proper, the dentate gyrus is composed of 3 layers. The cell bodies of the principal neurons, granule cells, form a densely packed V-shaped layer, enclosing the polymorphic cell layer, and interlocking with the hippocampus proper. The granule cell bodies lie deep to a third relatively cell free layer, the molecular layer, which comprises the apical dendrites of the granule cells and various afferent terminals, including the terminals of perforant path fibres from the entorhinal

cortex. The polymorph layer consists of non-granular interneurons, and is the zone in which granule cell axons come together to form the mossy fibre projection to area CA3.

The dentate gyrus is often divided into two portions. The suprapyramidal blade refers to the portion adjacent to area CA1, and the infrapyramidal blade refers to the opposite portion.

2.2.4 Extrinsic connectivity of the hippocampal formation

The major sensory input to the hippocampus is provided by the entorhinal cortex (see figure 2.2A & B). The entorhinal cortex receives highly processed multimodal information from several regions of association cortex, as well as direct input from the primary olfactory cortex. This information is conveyed to the hippocampus via the perforant path. This projection arises in layer II and III of the entorhinal cortex, and forms the dense "angular bundle" upon entering the hippocampus. The perforant path can be sub-divided into medial and lateral components, which terminate in the middle one-third and outer one-third of the dentate molecular layer respectively. Although the dentate gyrus is the major target of the perforant path, projections to all subfields of Ammon's horn and the subiculum have been identified.

In addition to the glutamatergic perforant path input, the hippocampus receives a number of projections from sub-cortical structures. For instance, a serotoninergic projection arises in the raphé nucleus and projects to the hippocampus via the fornix, and a noradrenergic projection is provided by the locus coeruleus. A cholinergic / GABA-ergic input arises from the medial septal nucleus, and nuclei of the diagonal band of Broca and projects to the hippocampus via the fornix. The septal input is believed to be the source of the hippocampal theta rhythm, a topic discussed in section 2.4.8.

The basolateral nucleus of the amygdala is known to project to the ventral portion of Ammon's horn. In addition, a noradrenergic projection from this amygdaloid nucleus to the dentate gyrus has been identified electrophysiologically, but not anatomically (Ikegaya et al., 1996, 1997).

2.2.5 Intrinsic connectivity of the hippocampal formation: the tri-synaptic circuit

The salient features of the intrinsic connectivity are often characterized as a tri-synaptic loop (figure 2.2A). The projections within this loop are almost exclusively unidirectional. The circuit starts with perforant path afferents which synapse onto granule cells of the dentate gyrus. The axons of these granule cells form the mossy fibre pathway, which makes *en passant* synapses with the dendrites of the CA3 pyramidal cells. The axons of the CA3 pyramidal cells form the Schaffer collateral pathway which projects to the CA1 pyramidal cells. In addition to forming the Schaffer collaterals, the axons of CA3 pyramidal cells divide and give rise to associational projections to other CA3 pyramidal cells,

as well as commissural projections to CA1 and CA3 pyramidal cells in the contralateral hippocampus. The pyramidal cells of CA1 project back to the entorhinal cortex, and indirectly via the subiculum.

As mentioned above, both CA1 pyramidal cells receive commissural projections from the contralateral CA3 region. In addition, the inner third of the molecular layer of the dentate gyrus receives a projection from both the ipsilateral and the contralateral polymorphic layer, known as the ipsilateral associational / commissural projection.

2.2.6 The lamellar hypothesis

On the basis of electrophysiological and neuroanatomical studies, it was proposed that the major excitatory pathways of the tri-synaptic loop run almost exclusively in a direction perpendicular to the septotemporal axis of the hippocampus, with a highly restricted lateral spread (Andersen et al., 1969, 1971). According to this view, the hippocampus is composed of a large number of semi-autonomous transverse slices, a structure reminiscent of the columnar organization of neocortex. However, Amaral and Witter (1989) have challenged this notion on the basis of new neuroanatomical evidence suggesting that with the exception of the mossy fibre pathway, many of the intrinsic hippocampal connections are as extensive in the septotemporal direction as in the transverse direction. Furthermore, different projection systems have distinct and highly topographically organized patterns of organization in the septotemporal axis.

Hence, the lamellar hypothesis in its original form may be an oversimplification. Nevertheless many important aspects of hippocampal physiology, including the expression of LTP, are captured in the *in vitro* hippocampal slice, a convenient experimental preparation in which transverse hippocampal sections of approximately 400 μm in thickness are maintained by bath perfusion with artificial CSF. Proponents of the lamellar hypothesis question whether slice preparations would work if a large part of the intrinsic circuitry were discontinuous as implied by Amaral and Witter (1989).

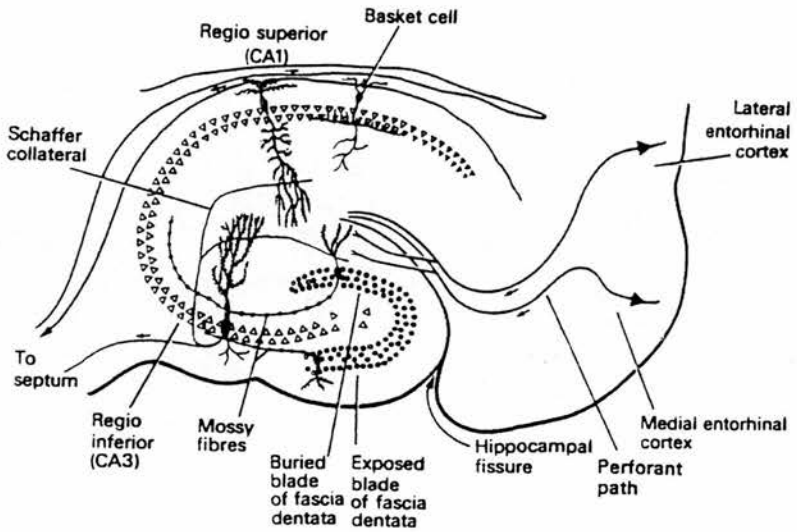
2.2.7 Efferent projections from the hippocampus

The subiculum provides the main output pathway from the hippocampus, giving rise to a prominent projection back to the entorhinal cortex, as well as projections to a range of neocortical areas. In addition, subicular efferents project prominently to a number of subcortical areas, including the septal complex, the mammillary bodies, the nucleus accumbens and the thalamus. As well as projecting via the subiculum, CA1 neurons project directly to a number of cortical areas, as well as the amygdala, hypothalamus and lateral septum.

2.2.8 Summary

The hippocampus receives highly processed polysensory information from associational areas of neocortex, together with various subcortical modulatory inputs, and projections from the amygdala that may carry informational about the motivational significance of sensory information. The output pathways of the hippocampus to neocortical, striatal and limbic regions are likely to be involved in influencing the behaviour of an animal in response to learning. For an extensive recent review of hippocampal anatomy, see Amaral and Witter (1995).

(A)



(B)

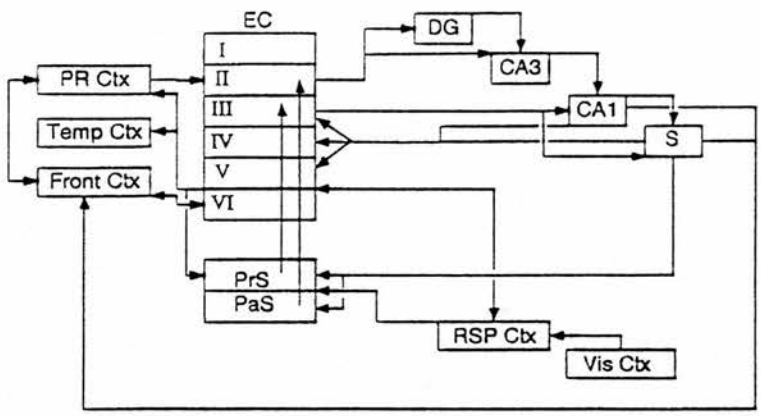


Fig. 2.2

(**A**) Horizontal section through the hippocampus. A schematic diagram of the intrahippocampal connections comprising the tri-synaptic loop (from O'Keefe and Nadel, 1978). (**B**) Major intrinsic connections of the hippocampal formation and major extrinsic cortical inputs. This diagram summarises current knowledge of both serial and parallel aspects of the intrinsic hippocampal circuitry such as the direct entorhinal projections to areas CA3 and CA1 not illustrated in (A). PR Ctx, perirhinal cortex; Temp Ctx, temporal cortex; Front Ctx, frontal cortex; Vis Ctx, visual cortex (from Amaral and Witter, 1995).

2.3 The function of the mammalian hippocampus

2.3.1 Introduction

The role of the hippocampus remains uncertain despite an intensive research effort over recent years, and the generation of numerous theories of hippocampal functioning. However, the hippocampus is undeniably involved in certain forms of spatial learning. A major issue in the literature concerns whether spatial memory represents a particular example of a broader class of declarative memory for which the hippocampus is required, or whether the hippocampus is preferentially concerned mainly with navigation and the recall of spatial locations. A systematic review of the literature on this topic is beyond the scope of this thesis, but a few salient areas will be briefly discussed.

2.3.2 Evidence from human case studies

The critical involvement of temporal lobe structures in memory was first revealed by the study of a now famous amnesic patient, "H.M." (Scoville and Milner, 1957). H.M. was left with severe amnesia following bilateral temporal lobe resection as a treatment for intractable epilepsy. The most striking aspect of H.M.'s impairment is an anterograde amnesia which leaves him unable to retain events in memory for more than a few minutes. In addition, he exhibits a limited retrograde amnesia extending back a few years before the surgery. H.M.'s I.Q. is normal, and his memory impairment is limited to "declarative" memory (see Squire, 1987), particularly the recall of personal events. However, H.M. exhibits intact "procedural" memory, and is, for instance, able to learn new motor skills (see Milner, 1962).

Evidence from other amnesic patients has confirmed the importance of the hippocampus in memory. Patient "R.B." became amnesic after an ischaemic episode. Post-mortem histological examination revealed that the damage was almost exclusively limited to the CA1 subfields of the hippocampus, suggesting that damage limited to the hippocampus alone can cause amnesia (Zola-Morgan et al., 1986).

2.2.3 Evidence from hippocampal lesions in primates and rats

The results of behavioural experiments on animals with hippocampal lesions have given rise to a large number of theories of hippocampal function. According to some of these, the hippocampus is involved in a particular form of information processing, regardless of the nature of that information. Such accounts include working memory theory (Olton et al., 1979; Wan et al., 1994), declarative

memory theory (Squire, 1992), and configural association theory (Sutherland and Rudy, 1989). Other theories have emphasized the type of information learned, for instance spatial (O'Keefe and Nadel, 1978; Morris et al., 1982, 1990b; Gaffan, 1991; Worden, 1992) or contextual (Hirsh, 1974; Honey and Good, 1993).

Early studies indicated that severe memory impairments in monkeys could be obtained by surgical removal of the hippocampus, amygdala, and adjacent cortical regions (Mishkin, 1978; Zola-Morgan et al. 1982; Murray and Mishkin, 1984). The behavioural test used in these studies was a delayed non-matching-to-sample (DNMS) object recognition task. A deficit in this task is generally characterized as an impairment of declarative memory (see Squire, 1987, 1992). Combined damage to the hippocampus, perirhinal and parahippocampal cortices has also been reported to cause a severe memory impairment (Zola-Morgan et al., 1993). However, lesions of the perirhinal and parahippocampal cortical areas alone have been found to produce an equally severe deficit, suggesting that the role of the hippocampus itself may be minimal (Zola-Morgan et al., 1989; Suzuki et al., 1993).

Nevertheless, radio-frequency lesions limited to the hippocampus, but sparing adjacent cortical areas have been reported to cause a modest impairment in DNMS performance (Alvarez et al., 1995). However, it has recently been reported that animals with axon-sparing excitotoxic lesions (see below) of the hippocampus and amygdala are unimpaired in DNMS performance (Murray and Mishkin, 1998). It is thought that conventional lesions may transect a number of efferent pathways from the perirhinal and entorhinal cortices. Since these regions are known to play a role in DNMS performance, it is possible that this inadvertent damage, rather than hippocampal ablation itself, was responsible for the DNMS deficit observed in earlier studies.

Differences in lesion technique have undoubtedly also contributed to the confusion surrounding the rodent literature. As in many primate studies, non-axon-sparing lesions have often been used. Conventional lesion techniques, such as aspiration, electrolytic or radiofrequency, cause damage to fibres of passage and to adjacent cortical areas, making the contribution of the hippocampus difficult to judge. However, the injection of small volumes of the excitotoxic agent ibotenic acid at numerous hippocampal sites results in the selective death of those neurons having their cell bodies within the dentate gyrus and the CA subfields only (Jarrard, 1989). Using this technique, performance is unimpaired on a number of tasks formerly thought to be hippocampus-dependent (See Jarrard, 1993 for review). However, robust impairments are still obtained on spatial learning tasks, and in tasks requiring the use of contextual cues (Jarrard et al., 1987; Morris et al., 1990b; Honey and Good, 1993). This latter result is reminiscent of the finding that fornix transection in primates impairs object discrimination learning, but only when successful performance depends on the processing of complex visual scenes (Gaffan and Harrison, 1989). In addition, it has recently been found that monkeys with

excitotoxic hippocampal lesions are impaired in a task that requires the animals to learn where to reach within two-dimensional spatial scenes (Murray et al., 1998).

The idea that the hippocampus is involved in the mapping of spatial location was first proposed by O'Keefe and Nadel (1978). The finding that certain principal hippocampal neurons fire selectively when a rat is in a certain spatial location (O'Keefe and Dostrovsky, 1971) added further evidence in addition to the hippocampal lesion literature available at the time. A number of studies have since implicated the activity of such "place cells" in spatial navigation (see Bures et al., 1997). The consequences of excitotoxic lesions described above would also seem to support the notion that the hippocampus is preferentially involved in the processing of spatial information.

However, a number of pieces of evidence are not consistent with this limited view of hippocampal function. To give one example, it was recently reported that rats with ibotenic acid lesions of the hippocampus are impaired in the learning of non-spatial odour association tasks when the flexible expression of memory is required (Bunsey and Eichenbaum, 1996). Specifically, rats were impaired in tests of transitivity (i.e. if A ⇒ B and B ⇒ C, then A ⇒ C), and symmetry (i.e. if B ⇒ C then C ⇒ B). Hippocampal lesioned rats were not impaired in simple paired associate learning. This result has been interpreted as suggesting that the hippocampus is, after all, a declarative memory structure. According to this view, spatial tasks are merely one example, albeit a very good one, of a class of declarative memory tasks for which the hippocampus is required.

2.3.4 The watermaze task as an index of hippocampal function

Despite the ongoing debate about the precise role of the hippocampus, lesioning this structure results in a profound spatial learning deficit in the watermaze, a piece of apparatus specifically designed to study spatial learning in rats (Morris, 1984; Stewart and Morris, 1993).

In a reference memory task in the watermaze, a rat must remember the fixed spatial location of a hidden escape platform on the basis of cues positioned in the room surrounding the maze. The use of random starting positions ensures that the platform cannot be located simply by swimming towards individual cues, or by repeating fixed patterns of body movement. During training, performance is assessed by measuring the latency to find the hidden platform. At the end of acquisition, a probe trial or "transfer test" is often carried out, in which the platform is removed and the rat allowed to swim freely for a limited period of time. Transfer test performance is conventionally assessed by measuring the percentage time spent in the quadrant of the pool that formerly contained the escape platform. Whilst the platform may be discovered fairly rapidly during acquisition trials, either by chance or by adopting a simple strategy such as swimming at a fixed distance from the pool walls, such factors cannot account for a bias towards the target quadrant during a transfer test. As a result, this measure is

generally considered to provide a more sensitive index of memory than escape latency. However, a transfer test is an extinction trial, and as such depends on the persistence of searching in the absence of reward. For this reason, transfer test performance, although arguably the best measure available, has the potential to be misleading under some circumstances. This issue is discussed further in chapter 6.5.

A robust deficit in both escape latency and transfer test performance is obtained in hippocampectomized rats, whether aspiration lesions or ibotenic acid lesions are used (Morris et al., 1982, 1986b and 1990b respectively). A number of non-spatial control tasks have been used to confirm that the deficit is limited to spatial learning. For instance, hippocampal lesions do not impair the ability of rats to locate and swim towards a visible platform (Morris et al., 1982). In addition, lesioned rats learn as rapidly as controls to discriminate between two visually distinct platforms, one of which is rigid and provides a means of escape, and the other of which is floating and will not support the weight of a rat (Morris et al., 1986b).

Owing to its exquisite sensitivity to the effects of hippocampal lesions, the watermaze has frequently been used to assess the behavioural effects of treatments designed to modulate putative hippocampal mechanisms of information storage. For this purpose, it is not important to know whether the role of the hippocampus is limited to spatial learning, or extends to a wider class of declarative memory functions. It is in this way, as a behavioural assay, that the watermaze is used in chapters 5 and 6 of this thesis.

2.4 The mechanisms of hippocampal long-term potentiation

2.4.1 Introduction

The notion that synaptic changes may underlie the formation of memories was first proposed over a hundred years ago by Ramón y Cajál, who suggested that learning might involve the experience-dependent growth of connections between neurons. In the middle of the present century, Konorski (1948) presented a theoretical proposal concerning the circumstances during which associative learning might arise. The essence of this proposal was that in order for an increase in associative strength to occur, activity in an "emitting centre" must be coincident with rising activity in a "receiving centre". The following year, an explicitly synaptic mechanism for associative learning was advanced by Hebb (1949). In this scheme, if a presynaptic cell persistently takes part in firing a postsynaptic target neuron, a change occurs in one or both cells such that the connection between the two cells is strengthened. Although these two proposals are not identical, both contain the suggestion that coincident pre- and postsynaptic activity is necessary for the enhancement of connections between neurons.

The significance of such a proposal is illustrated by the hypothetical case in which one presynaptic neuron weakly excites a postsynaptic cell at the same time as a second presynaptic afferent strongly activates and perhaps fires the cell. According to the above condition of coincident of pre- and postsynaptic activity, the strength of the connection between the initially weak input and its postsynaptic target should be strengthened. Note that in this case, each input is now able to strongly activate the postsynaptic cell. Computational modelling of the activity in networks of neuron-like elements obeying rules similar to the above has given rise to an entire research field in its own right (see Gluck and Rumelhart, 1990). For examples of hippocampal neural network modelling, see Rolls et al. (1997) and Treves and Rolls (1994).

2.4.2 The discovery of hippocampal long-term potentiation

In the late 1960s, an electrophysiological phenomenon was discovered that appeared to fit the requirements for a cellular learning mechanism such as that postulated by Hebb (1949). The phenomenon of long-term potentiation was initially discovered by Lømo in 1966, but was first reported in detail seven years later (Bliss and Lømo, 1973).

In these studies, carried out in anaesthetized rabbits, electrical stimulation was delivered to the perforant path from the entorhinal cortex, and evoked field potentials were recorded from the synaptic, or molecular, layer of the dentate gyrus. At this point, a negative-going EPSP is recorded,

reflecting the flow of positive ions into the dendrites in response to the electrical stimulation of afferent fibres. Superimposed on the EPSP is a positive-going population spike reflecting the synchronous firing of dentate granule cells.

Bliss and Lømo (1973) first recorded a "baseline" during which perforant path stimulation was delivered every 2-3 s, after which a high frequency conditioning train, often known as a tetanus, was delivered to the perforant path. Upon resuming stimulation at the initial low frequency, the size of the EPSP was observed to be greater. Both the amplitude of the EPSP and the amplitude of the superimposed population spike were found to be increased. The population spike latency was also reduced. These changes lasted up to 10 hours in the acute preparation, and subsequent studies in animals with chronically implanted electrodes revealed that the phenomenon could last for a period of several weeks (Bliss and Gardner-Medwin, 1973).

A similar form of LTP was subsequently discovered in the Schaffer collateral-commissural input to CA1 *in vitro* (Alger and Teyler, 1976; see section 2.2.6). LTP has since been reported in other brain regions, including the neocortex (e.g. Artola and Singer, 1987). However, the following discussion is limited to the properties and mechanisms of hippocampal NMDA receptor-dependent LTP only.

2.4.3 Properties of LTP

Since its discovery, LTP has been found to have a number of properties that make it an attractive candidate mechanism for the formation of memories. "Associativity" describes the finding that the pairing of a weak, sub-threshold input with a strong suprathreshold input can lead to a potentiation of the weak input (McNaughton et al., 1978; Levy and Steward, 1979). A related property is that of cooperativity, referring to the mutual facilitation between independent afferent inputs. It has been reported that the more fibres activated during tetanization, the more LTP is obtained, owing to the cooperative interaction between inputs (McNaughton et al., 1978; Larson and Lynch, 1986). A third property, input-specificity, refers to the fact that only those synapses that are active during the LTP induction event become potentiated (Andersen et al., 1977; Gustafsson et al., 1987; but see Engert and Bonhoeffer, 1997). Potentiation that exhibits the above properties is sometimes described as "hebbian".

2.4.4 The mechanisms of LTP induction

The main excitatory neurotransmitter in the mammalian hippocampus is L-glutamate (Watkins and Evans, 1981). Fast excitatory transmission is mediated by the action of glutamate on ionotropic

receptors of the AMPA subtype. When activated, the AMPA receptor allows the passage of sodium ions through its ionophore, thus depolarizing the cell.

Another class of ionotropic glutamate receptor, the NMDA receptor, consists of a glutamate binding site and a voltage-gated ionophore. NMDA receptors allow the passage of Ca^{2+} and Na^{2+} ions into the cell only during glutamate binding coupled with postsynaptic depolarization. At the normal resting potential, the ionophore is blocked by the presence of Mg^{2+} ions. However, postsynaptic depolarization releases the Mg^{2+} block, and when paired with glutamate binding, Ca^{2+} flows through the ionophore (Nowak et al., 1984). The dual ligand and voltage gated action of the NMDA receptor means that Ca^{2+} entry only occurs under circumstances in which presynaptic glutamate release is paired with postsynaptic depolarization. Hence, the NMDA receptor embodies at the molecular level the mechanisms postulated in section 2.4.1.

The involvement of NMDA receptors in hippocampal LTP was first revealed by the application of the NMDA receptor antagonist D,L-AP5 to a CA1 slice preparation (Collingridge et al., 1983; Harris et al., 1984). This had little effect on the EPSP evoked by low frequency stimulation of the Schaffer collateral pathway, but prevented the induction of LTP following a high frequency tetanus. A later study showed that the application of AP5 after tetanization had no effect on the level of potentiation, demonstrating that NMDA receptors are required only for the induction, but not the maintenance of LTP (Davies and Collingridge, 1989). It has since been demonstrated that intraventricular or direct intrahippocampal infusions of AP5 can block the induction of dentate LTP *in vivo* (Morris et al., 1986a; Errington et al., 1987; Morris, 1989). Note that while the above studies show that NMDA receptors play a necessary role in LTP induction, they do not address the question of whether NMDA receptor activation is in itself sufficient. This issue is discussed in chapter 3.10.1.

During low frequency synaptic transmission, glutamate is released and binds to both AMPA and NMDA receptors. The subsequent AMPA receptor activation causes an inward sodium current, and cumulative activity at many synapses may result in postsynaptic depolarization, a situation that would appear to be sufficient to cause NMDA receptor activation. However, the activation of GABA-ergic interneurons rapidly repolarizes the cell, before the threshold for NMDA receptor activation is reached. The role of $GABA_A$ receptors in LTP induction was first revealed by Wigström and Gustafsson (1983), who found that bathing hippocampal slices in the $GABA_A$ antagonist picrotoxin facilitated the induction of LTP, owing to an increase in postsynaptic depolarization. During tetanization, a frequency-dependent depression of inhibitory transmission is observed, a result attributed to a presynaptic $GABA_B$ receptor-mediated reduction in GABA release (Davies et al., 1991). This reduction in inhibition allows further postsynaptic depolarization, and hence the opening of the NMDA receptor channel, to proceed.

2.4.5 Intracellular second messengers involved in LTP induction and expression

As mentioned above, the activation of NMDA receptors causes an influx of calcium into the postsynaptic cell, an event that is believed to trigger a number of intracellular signalling cascades leading to the expression of LTP. Early evidence that calcium is involved in LTP was provided by the finding that injection of a calcium chelator, EGTA, into the postsynaptic cell prevented the induction of LTP (Lynch et al., 1983). Calcium plays a central role in almost all forms of intracellular signalling, and many of its targets are enzymes that trigger the production of other second messengers. There is now evidence that the release of calcium from intracellular stores in addition to calcium influx via the NMDA ionophore is necessary for the induction of LTP (see chapter 3.10).

The calcium activated kinase, protein kinase C (PKC) has been implicated in LTP by a number of studies indicating that inhibitors of this enzyme block the induction of LTP, whilst sparing a short-term potentiation lasting 20 to 90 min (Lovinger and al., 1987; Reymann et al., 1988 a, b; Malinow et al., 1988). Inhibitors of the calcium / calmodulin-dependent protein kinase II (CaMKII) also block LTP induction (Ito et al., 1991) as do calmodulin inhibitors (Reymann et al., 1988b; Malenka et al., 1989). Furthermore, mice lacking the gamma isoform of PKC, or the alpha-subunit of CaMKII are deficient in LTP (Abeliovich et al., 1993a; Silva et al., 1992a).

In addition to its direct actions on PKC and CaMKII, calcium stimulates the production of cAMP in conjunction with calmodulin, by activating a calmodulin sensitive form of adenylate cyclase (Chetkovich and Sweatt, 1993). However, the activation of dopaminergic inputs during strong tetanization is also believed to be necessary in adult animals (Frey and Morris, 1998). The production of cAMP leads to the activation of protein kinase A (PKA), a kinase implicated in the maintenance of LTP. The application of PKA inhibitors within 1 hr of tetanization blocks a late phase of LTP that normally develops 3-4 hr after tetanization (Frey et al., 1993; Matthies and Reymann, 1993). In addition to a possible role in up-regulating AMPA receptor function (see below), PKA activates a transcription factor, the cAMP response element binding protein (CREB) that interacts with the cAMP response elements associated with certain genes, leading to their transcription (Yamamoto et al., 1988). A role for gene expression in late LTP is suggested by the fact that the persistence of LTP is substantially reduced by inhibitors of protein and mRNA synthesis (Frey et al., 1988; Abraham and Otani, 1991; Nguyen et al., 1994; see also Frey and Morris, 1997).

Several other classes of kinase have also been implicated in LTP, such as tyrosine kinases (O'Dell et al., 1991; Grant et al., 1992) and members of the mitogen activated kinase (MAPK) family (Bading and Greenberg, 1991), a detailed description of which is beyond the scope of this thesis.

One mechanism by which LTP might be expressed is the up-regulation of postsynaptic glutamate receptor function by phosphorylation (see section 2.4.6). For instance, CaMKII, and to a lesser extent PKC, can directly phosphorylate the AMPA receptor (McGlade-McCulloh et al., 1993), and the

application of PKA has been shown to cause an up-regulation of AMPA receptor functioning (Greengard et al., 1991). In addition, an up-regulation of NMDA receptor function is believed to result from phosphorylation by PKC (Ben-Ari et al., 1992).

2.4.6 The mechanisms and locus of LTP expression

Although the induction of LTP is known to be controlled postsynaptically, and almost certainly involves the activation of the intracellular signal transduction pathways discussed above, there is still uncertainty about the locus of expression of LTP. For example, LTP could be expressed simply by an up-regulation of AMPA receptor functioning, perhaps owing to phosphorylation by protein kinases, as mentioned above. Alternatively, an increase in the number of AMPA receptors might occur, or non-functional receptors might be converted from a "silent" to an "active" state (Kullmann, 1994; Liao et al., 1995). However, it is equally possible that LTP is expressed by a sustained increase in glutamate from the presynaptic terminal (Dolphin et al., 1992). Despite an intensive research effort over the past few years, the locus of LTP expression remains uncertain.

Early studies failed to reveal an increase in the postsynaptic response to glutamate application after tetanization, as was predicted by a postsynaptic locus of expression (e.g. Lynch et al., 1976). However, Davies et al. (1989) found that the response of CA1 neurons to AMPA gradually increased after LTP induction, reaching a peak roughly 2 hr after tetanization. The slow onset of this effect may explain the negative results reported in previous studies. Further evidence suggesting a postsynaptic locus was provided by the observation that following LTP induction, a selective increase was obtained in the AMPA receptor-mediated component of the EPSP, whilst the NMDA-receptor mediated component remained unchanged (Muller and Lynch, 1988; Muller et al., 1988; Kauer et al., 1988b; Kullmann, 1994). However, other groups have reported that both NMDA and AMPA-mediated components of the EPSP can be equally increased following LTP induction, a result that is consistent either with an identical postsynaptic increase in AMPA and NMDA receptor-mediated currents, or with a presynaptic locus of expression (e.g. Clark and Collingridge, 1995). It is thought that differences in the LTP induction protocol (i.e. pairing of presynaptic activity with postsynaptic depolarization *versus* tetanization of afferents) may explain the discrepancies in data obtained by different groups. However, the interpretation of such experiments is further complicated by the suggestion that NMDA receptors may, under some circumstances, be activated by glutamate "spill-over" from adjacent synapses (Kullmann et al., 1996).

An alternative approach is to attempt to measure increases in glutamate release from the presynaptic terminal, as predicted by a presynaptic locus of LTP expression. Using a variety of techniques, a number of groups have reported an increase in glutamate release after the induction of LTP (e.g. Dolphin et al., 1982; Bliss et al., 1986, 1990; Bekkers and Stevens, 1990a; Ghijsen et al., 1992).

However, for unknown reasons, others have failed to observe an increase in glutamate release after tetanization (Aniksztejn et al., 1989; Diamond et al., 1998; Lüscher et al., 1998). A further line of evidence for a presynaptic contribution to LTP has been provided by studies of paired pulse facilitation (PPF). This is generally regarded as a presynaptic phenomenon in which the response to the second of a pair of pulses is enhanced when the pulses are delivered in quick succession. This effect is believed to be caused by an increase in transmitter release in response to elevated presynaptic calcium levels following the first pulse. Despite negative results in initial studies (e.g. McNaughton, 1982), changes in PPF have been reported during the first hour of LTP, suggesting that the induction of early LTP, at least, may involve a change in the presynaptic machinery involved in neurotransmitter release (e.g. Christie and Abraham, 1994; Schultz et al., 1994; Kleschevnikov, 1997). However, the suitability of PPF as an index of presynaptic release probability is questionable. For instance, the PPF of NMDA receptor-mediated EPSCs has been found to be strongly dependent on postsynaptic voltage, suggesting the involvement of postsynaptic mechanisms (Clark et al., 1994).

The debate concerning the locus of expression of LTP is far from being resolved. However, an issue that arises from the possibility of a presynaptic mechanism of LTP expression concerns the need for retrograde signalling between the postsynaptic terminal at which LTP induction occurs, and the presynaptic terminal. The nature of this signal, presumed to be chemical, is currently uncertain. While a number of small molecules have been implicated in this role, including nitric oxide, carbon monoxide, and arachidonic acid, conclusive evidence is not available for any of these candidates, and the nature of the retrograde messenger remains a controversial topic (for reviews see Williams, 1996; Medina and Izquierdo, 1995).

2.4.7 Phases of LTP

Analysis of the time course of LTP suggests that more than one underlying process is probably involved. Following a brief tetanus, LTP (or STP; see following paragraph) develops within approximately 30 s (Gustafsson and Wigström, 1990). However, strong tetanic tetanization results in a superimposed "post-tetanic potentiation" (PTP), believed to be a pre-synaptic phenomenon similar to paired-pulse facilitation. PTP is thought to result from an increase in release probability in response to a transient elevation of calcium levels in the presynaptic terminal following tetanization (see Zucker, 1993).

In parallel with this increase, a longer lasting NMDA-receptor dependent component, usually known as short-term potentiation (STP) becomes evident. Opinions differ as to the duration of this phase, and the mechanistic distinction between STP and LTP is similarly unclear. For instance, STP is often spared by protein kinase inhibitors that block LTP (see section 2.4.7). However, other evidence suggests that STP might be affected to the same extent as LTP by PKC inhibition (Hanse and

Gustafsson 1994). It is possible that STP and LTP represent a continuum of plastic changes of variable duration, depending on the strength of the induction parameters. Nevertheless, it has recently been reported that STP and LTP do not occlude each other, and have differential effects on paired-pulse facilitation, suggesting that the two forms of plasticity may indeed be expressed via different mechanisms (Schulz and Fitzgibbons, 1997). This debate has still not been satisfactorily resolved.

The induction of LTP is, as described above, dependent on the activation of protein kinases. However, LTP is often defined in more pragmatic terms as potentiation lasting for 1 hr or more. However, a distinct, late phase of LTP (late LTP or L-LTP) has been identified by studies showing that the application of protein and mRNA synthesis inhibitors limits the duration of LTP to approximately 4 hr (Krug et al., 1984; Frey et al., 1988; Otani and Abraham, 1989; Nguyen et al., 1994; Frey et al., 1996). The expression of both early and late forms of LTP may involve structural changes at potentiated synapses (e.g. Buchs and Muller, 1996; Geinisman et al., 1996; Voronin et al., 1995).

2.4.8 The role of the hippocampal theta rhythm in synaptic plasticity

The hippocampal theta rhythm is manifested as a large amplitude, approximately sinusoidal EEG oscillation of between 5-12 Hz in the behaving rat (see Vanderwolf et al., 1975; Vinogradova, 1995 for review). Hippocampal complex spike cells have been observed to fire short bursts of action potentials at a specific phase of the theta rhythm (Fox et al., 1986). In area CA1 of freely moving rats, there is evidence that bursting activity is associated with significant behavioural events in an olfactory discrimination task, and a spatial learning task (Otto et al., 1991).

A number of studies have reported that LTP is optimally induced using stimulation that mimics the firing patterns associated with the endogenous theta rhythm. For instance, the delivery of short bursts of 100 Hz stimulation at intervals of 200 ms results in very robust LTP lasting for several weeks *in vivo* (Larson et al., 1986; Rose and Dunwiddie, 1986; Stäubli and Lynch, 1987). It was subsequently found that a single priming pulse followed 140-200 ms later by a short burst of 2-10 pulses could induce stable LTP, referred to as "primed burst" potentiation (Diamond et al., 1988). It has been suggested that the occurrence of a priming pulse 170-200 ms prior to a burst of high frequency stimulation suppresses feed-forward inhibition and leads to the prolongation of EPSPs, which in turn leads to substantial NMDA receptor activation (Arai and Lynch, 1992). A similar mechanism might explain the robust LTP induced by theta burst stimulation.

It is found that LTP induction is facilitated in the presence of hippocampal theta (Bramham and Srebro, 1989; Huerta and Lisman, 1993). It has also been reported found that LTP is preferentially induced by burst stimulation on the positive phase of the theta rhythm in urethane-anaesthetized rats

(Pavlides et al., 1988). Stimulation on the negative phase of theta had no effect, or resulted in a depression of population spike amplitude. A similar result has subsequently been reported in CA1 slices bathed in carbachol in order to elicit a theta rhythm. The delivery of trains of single shocks each locked to a positive theta peak was sufficient to induce LTP, whilst stimulation on the negative phase had no effect, or occasionally induced LTD (Huerta and Lisman, 1993). It was subsequently discovered using the same preparation that the delivery of either a single burst or a train of single pulses phase-locked to the negative phase of theta could reliably induce a depotentiation of existing LTP (Huerta and Lisman 1995, 1996a, b). Similarly, burst stimulation in CA1 *in vivo* has been reported to result either in LTP or depotentiation, depending on its phase relationship to the tail pinch-triggered theta rhythm (Hölscher et al., 1997c).

These results suggest that the hippocampal theta rhythm may be involved in bi-directional modifications of synaptic strength under physiologically plausible conditions (see chapter 8.5.1).

2.5 Long-term depression (LTD) of synaptic efficacy

2.5.1 Classes of LTD

Several forms of hippocampal LTD have been identified, including heterosynaptic LTD, homosynaptic LTD and associative LTD. Associative LTD has been induced by the "anti-hebbian" pairing of single pulses in the test pathway with the delivery of short high frequency trains to an independent input (Stanton and Sejnowski, 1989). However, attempts to replicate these original findings have generally not been successful (Paulsen et al., 1993; Kerr and Abraham, 1993; but see Christie and Abraham, 1992). Heterosynaptic LTD is induced in inactive pathways following activation of the postsynaptic neuron, or orthodromic activation of a separate input to the same neuron. It was first observed as a correlate of LTP in area CA1 *in vitro* (Lynch et al., 1977), but is readily induced in the dentate gyrus *in vivo* (Levy and Steward, 1979; Abraham and Goddard, 1983). The following section will concentrate entirely on homosynaptic LTD, the depression of synaptic transmission in a single input pathway activated by low frequency stimulation. The related phenomenon of homosynaptic depotentiation is discussed in chapter 8.1.

2.5.2 Induction of homosynaptic LTD in area CA1 in vitro

A number of studies have revealed that low frequency activation of the Schaffer collateral / commissural fibres to area CA1 *in vitro* can lead to a lasting homosynaptic depression of synaptic efficacy, known as long-term depression (LTD). The effect was first reported by Dunwiddie and Lynch (1978) using 100 pulses at 1 Hz, although the depression that they observed was only recorded for 5 min, and was not input specific. Later studies reported that the delivery of 900 pulses at 1-3 Hz induces a sustained, input specific LTD (Dudek and Bear, 1992, 1993). Similar results have been reported by many others (e.g. Mulkey and Malenka, 1992; Mulkey et al., 1993, 1994; Bolshakov and Siegelbaum, 1994; Stevens et al., 1994; Kerr and Abraham, 1995; Wagner and Alger, 1995). Like LTP, LTD is generally found to be saturable, reversible, and NMDA receptor-dependent.

Despite the fact that homosynaptic LTD was first reported in slices from adult rats (Dudek and Bear, 1992), most subsequent studies have used slices from immature animals. In fact, a number of groups have reported a failure to induce homosynaptic LTD in slices from adult rats, suggesting that the phenomenon may be of developmental significance only (Fujii et al., 1991; O'Dell and Kandel, 1994; Bortolotto et al., 1994; Bashir and Collingridge, 1994; Otani and Connor, 1995). The age-dependence of CA1 LTD has been attributed to a strengthening of $GABA_A$-mediated inhibition during development, and hence a reduction in NMDA receptor activation during low frequency stimulation (Wagner and Alger, 1995).

2.5.3 Induction of homosynaptic LTD in area CA1 in vivo

Considerable controversy surrounds the induction of homosynaptic LTD in area CA1 in adult rats *in vivo* using trains of low frequency stimulation. LTD has been successfully induced in this way by two groups (Heynen et al., 1996; Manahan-Vaughan, 1997; Manahan-Vaughan and Reymann, 1997b; see chapter 9.4.4), but most report little or no effect (Barrionuevo et al., 1980; Stäubli and Lynch, 1990; Thiels et al., 1994; Errington et al., 1995; Doyère et al., 1996; Stäubli and Scafidi, 1997; Doyle et al., 1997). In fact, Errington et al. (1995), were only able to obtain LTD and depotentiation in the CA1 region of very young (10-11 day old) animals.

However, Thiels et al. (1994) found that LTD could be induced under urethane anaesthetic *in vivo* by the delivery of low frequency (0.5 Hz) trains of paired pulses with a 25 ms inter-pulse interval. This form of LTD was NMDA receptor-dependent and reversible. It was suggested that the efficacy of the paired pulse protocol depends on the use of an inter-pulse interval equal to the delay of feedforward and feedback inhibition. Hence, the EPSP generated by the second pulse of a pair coincides with the IPSP generated by the first pulse, a circumstance proposed to result in LTD. This protocol has since been found to induce LTD in area CA1 of freely moving rats (Doyère et al., 1996).

Another method of successfully inducing LTD in area CA1 of adult rats *in vivo* has recently been reported by Xu et al. (1997). The authors found that the exposure of rats to a stressful situation, a procedure known to inhibit LTP (see chapter 9.4.5), facilitated the induction of LTD. This effect is thought to be mediated by glucocorticoid receptor activation (Xu et al., 1998a).

2.5.4 LTD of perforant path–dentate gyrus synapses

LTD is less readily induced in the dentate gyrus than in area CA1. Low frequency stimulation has been reported to induce LTD in dentate slices from young rats (O'Mara et al., 1995a, b; Trommer et al., 1996; Huang et al., 1997; Wang et al., 1997). This form of LTD has been reported to be NMDA receptor independent, but dependent on the activation of group II mGluRs (Huang et al., 1997) and calcium influx via low voltage activated calcium channels, together with the release of calcium from intracellular stores (Wang et al., 1997; O'Mara et al., 1995b). However, low frequency stimulation does not generally induce LTD in the dentate gyrus *in vivo*, either in immature (Errington et al., 1995) or adult rats (Abraham et al., 1996).

In addition to trains of single pulses, the paired pulse protocol used with success in area CA1 *in vivo* fails to induce LTD in the dentate gyrus *in vivo* (Doyère et al., 1996; Thiels et al., 1996). However, a modified protocol involving the delivery of pairs of two-pulse bursts (inter-pulse interval = 2.5-5.0 ms; inter-pair interval = 15-25 ms) at a low frequency (0.5-2 Hz) resulted in a robust and lasting LTD (Thiels et al., 1996). The authors suggested that the use of very short inter-pulse intervals might have

increased the NMDA receptor-mediated component of the EPSP (see Blanpied and Berger, 1992), without compromising the paired pulse inhibition induced by the inter-pair interval of 15-25 ms. This form of LTD was investigated in rabbits rather than rats, but could be induced both under anaesthetic or in the freely moving state, and was NMDA receptor-dependent.

2.5.5 Mechanisms of LTD induction and expression

Mulkey and Malenka (1992) found that postsynaptic calcium entry via NMDA receptors was necessary for the induction of LTD. Other suggested sources of calcium entry include mGluR-mediated calcium release from intracellular stores (Stanton et al., 1991), L-type Ca^{2+} channels (Bolshakov and Siegelbaum, 1994) and low voltage-activated Ca^{2+} channels (Wang et al., 1997).

It is well known that a large increase in calcium entry, such as that resulting from a high frequency tetanus, is necessary for the induction of LTP, perhaps owing to the activation of Ca^{2+}-dependent protein kinases (see section 2.4.5). It has been proposed that a modest increase in postsynaptic calcium levels, such as that resulting from low frequency afferent stimulation, causes LTD by the selective activation of phosphatases (Lisman, 1989; Artola and Singer, 1993). In support of this idea, the application of protein phosphatase inhibitors has been found to block the induction of LTD (Mulkey et al., 1993, 1994) and depotentiation (O'Dell and Kandel, 1994).

However, a recent study used the photolysis of caged Ca^{2+} compounds to investigate the calcium levels necessary to trigger LTP and LTD. No differences in the threshold for the induction of these two processes were observed (Neveu and Zucker, 1996b). Nevertheless, fluorescence imaging of postsynaptic calcium levels has revealed that low frequency stimulation induces a moderate calcium influx, and causes LTD, whilst high frequency stimulation causes a larger calcium influx and LTP (Schexnayder et al., 1997). Furthermore, it was found that manipulating the calcium influx shifted the frequency-dependence of LTP and LTD. For instance, tetanization at 100 Hz normally resulted in LTP, but in the presence of an L-type Ca^{2+} channel blocker, or low concentrations of AP5, a more modest increase in intracellular calcium was observed and LTD was obtained.

These contradictory results suggest that another factor is involved in the response to calcium during afferent stimulation, compared to the situation resulting from intracellular calcium release by the photolysis of caged chelators. This factor may be the spatial pattern of intracellular calcium changes in response to afferent stimulation. It is likely that calcium influx and release from intracellular stores occurs in a highly compartmentalized and regulated fashion. The notion that modest global calcium increases result in LTD, whereas large increases result in LTP, although supported by pharmacological and imaging studies, may be a great oversimplification of the truth.

One piece of evidence suggesting that the expression mechanisms of LTP and LTP share a common target is provided by the fact that levels of protein kinase Mζ (PKMζ, a constitutively active fragment of PKC) are increased following LTP induction, but decreased following LTD (Hrabetova and Sacktor, 1996). However, data on the mechanisms and locus of expression of LTD are currently inconclusive, and will not be discussed further.

2.6 The relationship between hippocampal synaptic plasticity and the cellular mechanisms of learning

2.6.1 Introduction

It is now widely assumed that LTP represents a memory mechanism, and numerous neural network models of learning are based on changes in connection strength between "neuron-like" elements. However, experimental support for a role of LTP-like processes in learning is mostly indirect and often inconclusive. The following sections provide a review of some of the approaches that have been used in an attempt to establish such a connection.

2.6.2 Learning-induced changes in synaptic efficacy

The most direct evidence for a link between LTP and learning would be provided by a demonstration that learning is accompanied by an increase in synaptic efficacy. However, there are a number of potential problems with this approach. In particular, it is likely that a learning-related change in synaptic strength, if such a phenomenon exists, will occur in a small proportion of hippocampal synapses only. Large changes in synaptic efficacy across whole populations of synapses, such as that observed after LTP induction, would not represent an efficient mechanism of information storage. Hence, naturally occurring LTP-like phenomena may be difficult to detect using field potential recordings.

Early studies reported an apparent transient increase in synaptic strength associated with exploratory behaviour in rats chronically implanted with stimulation and recording electrodes (Sharp et al., 1989; Green et al., 1990). During exploration, a rise in EPSP slope was recorded, but values gradually returned to baseline levels after rats were returned to their home cages. Paradoxically, however, the population spike amplitude decreased during exploration. Nevertheless, others have failed to observe learning-related changes in the EPSP (e.g. Cain et al., 1993)

A possible explanation for the initial positive results was suggested by Moser et al. (1993a), who monitored brain temperature as well as field EPSPs in behaving rats. It was found that exploratory activity was accompanied by a rise in brain temperature that paralleled the EPSP rise, and the fall in population spike amplitude. Similar changes could be induced merely by passively warming the rats. Note that a temperature increase causes a fall in population spike amplitude, a result attributed to the reduced amplitude of individual action potentials, and a decrease in temporal summation (see Andersen and Moser, 1995).

However, after controlling for the temperature artifact, Moser et al. (1993b) identified a small temperature-independent increase in the fEPSP during exploration. This component increased at the start of exploratory activity, but fell back to baseline within about 15 min. This residual potentiation may reflect a true learning-related increase in synaptic efficacy, although the effect was transient, and may be an artifact of temperature-independent but movement-related neuromodulatory influences.

The measurement of changes in the fEPSP during exploration has not provided a definitive answer to the question of whether learning results in increases in synaptic strength. An alternative approach has been to investigate not whether behaviour induces LTP-like changes, but whether behaviour can modulate existing LTP. For instance, Seidenbecher et al. (1995) investigated the effects of drinking 1 hr after a tetanus in water-deprived rats and non-deprived controls. The LTP induced by a strong tetanus was not affected by drinking. However, the LTP resulting from a weak tetanus, not normally sufficient to induce lasting potentiation, was facilitated by drinking in the water-deprived group only. Subsequent work indicates that the hippocampal signal provided by drinking is noradrenergic in nature (Seidenbecher et al., 1997). This result suggests that a motivationally significant event can "rescue" LTP that under normal circumstances would have decayed back to baseline. If changes in synaptic efficacy do indeed underlie learning, then such a mechanism may serve to preserve motivationally significant information which might otherwise be lost.

2.6.3 The effects of LTP saturation on learning

If changes in synaptic strength underlie the acquisition of new information, it should be possible to block learning by driving synaptic strengths throughout the hippocampus to their maximum values. Numerous studies have attempted to achieve this by saturating hippocampal LTP. It is worth noting, however, that such an approach may be theoretically impossible. It is likely that the induction of a large increase in synaptic efficacy will engage mechanisms designed to protect against excessive excitation and epileptiform activity. Such protective mechanisms might include the occurrence of LTD at some synapses, in conjunction with LTP at others. As such, a true saturation of LTP may be unattainable.

It was initially reported that the saturation of dentate LTP with a course of high frequency tetanization impaired the performance of rats in a circular platform task requiring the learning of a goal location on the basis of extramaze cues (McNaughton et al., 1986). Similar results were subsequently obtained in the watermaze (Castro et al., 1989). In this study, an additional group of rats, tested after the decay of LTP back to baseline, learned normally. This result suggested that the impairment was unlikely to be a result of permanent damage caused by repeated tetanization.

However, subsequent attempts to replicate these findings, including one attempt from McNaughton's own laboratory, have been unsuccessful (McNamara et al., 1992; Cain et al., 1993; Jeffery and Morris, 1993; Korol et al., 1993; Sutherland et al., 1993). The reason for this discrepancy is unknown. It is possible that the small number of animals used in the Castro et al. (1989) study resulted in a false positive result.

The failure to obtain a block of learning in the above studies does not necessarily imply that saturation of LTP has no effect on learning. For instance, tetanization at a single site within the perforant path may fail to saturate LTP throughout the entire septotemporal extent of the hippocampus, leaving sufficient residual plasticity for normal learning. Alternatively, the asymptotic level of LTP regarded as the "saturation" point may not reflect the true maximal level of synaptic efficacy attainable. The use of different tetanization parameters may result in a different maximal level of LTP (see Jeffery et al., 1993).

With respect to the first of the possibilities raised above, it has been reported that LTP saturation contralateral to a unilateral hippocampal lesion does indeed cause a watermaze spatial learning deficit (Mumby et al., 1993). In a recent study, recording electrodes were implanted into the dorsal dentate gyrus of rats which had previously received a complete hippocampal lesion contralateral to the recording site (Moser et al., 1998). A course of tetanic stimulation was delivered via two bipolar stimulating electrodes straddling the perforant path. Saturation of LTP was assessed by tetanization at a third stimulating site. In these animals, LTP saturation resulted in an impairment in the acquisition of a watermaze spatial reference memory task, a result that provides some evidence that driving synaptic strengths to their asymptote may indeed prevent further learning.

Such results must be interpreted with some caution. A widespread increase in synaptic efficacy represents a potentially epileptogenic event. It is difficult to dissociate the role of LTP saturation on learning from the possible role of compensatory responses to repeated tetanization, or the role of neuroprotective mechanisms engaged by such activity. The demonstration that spatial learning is normal after the decay of LTP would not provide sufficient evidence to counter such a claim, since compensatory changes might also have subsided by this time. Hence, although the results of Moser et al. (1997) provide arguably the strongest evidence to date, the link between LTP capacity and learning capacity remains controversial.

2.6.4 Correlations between the magnitude of LTP and learning ability

If both learning and LTP depend on the same mechanisms of synaptic plasticity, it is possible that the saturated level of LTP in an individual rat may provide a general measure of the modifiability of

synapses within that animal. On this basis, it might be predicted that the level of LTP would correlate with learning ability, even if LTP saturation does not actually impair learning.

Such an association has indeed been reported by some researchers. For instance, Barnes (1979) found a significant positive correlation between the level of dentate LTP induced by three daily sessions of tetanization, and performance on the circular platform task mentioned in section 2.6.3. Young rats were found to learn the task faster and with fewer errors than old rats, a difference that paralleled the greater level of LTP induced in young rats after three days of tetanization. This difference in LTP was attributed to a faster rate of decay between sessions in old rats. In a subsequent study, it was found that the decay of LTP in both young and old rats paralleled forgetting as assessed by retention performance (Barnes and McNaughton, 1985). Young rats remembered the task for longer and showed more persistent LTP.

Consistent with this result, it was observed that in brain slices taken from rats previously trained in the watermaze, the amount of CA1 LTP obtained after tetanization correlated with watermaze performance (Kleschevnikov and Marchbanks, 1993). A similar result was reported by Jeffery and Morris (1993) who found that the saturated level of dentate LTP in freely moving rats correlated with subsequent watermaze performance. However LTP saturation did not itself impair learning. Curiously, the correlation between LTP and performance was positive at high test pulse intensities, but negative at low test pulse intensities. The reason for this paradoxical result is unknown (but see Jeffery, 1995, 1997 for discussion).

However, the finding that LTP correlates with spatial learning does not prove that the two phenomena are causally related. For instance, variability in a third factor that influences both LTP and learning in a similar way might lead to a spurious correlation between the two variables. An example of such a third variable is stress, a factor known to impair hippocampal LTP (e.g. Foy et al., 1987; see chapter 9.4.5) and learning (see McEwen and Sapolsky, 1995 for review). Hence, correlational data of the type presented above provide only circumstantial evidence for the involvement of LTP-like mechanisms in learning.

A slightly different approach is represented by attempts to identify biochemical changes associated with both LTP and learning. For instance, an increase in presynaptic glutamate release has been reported following both LTP (see section 2.4.6) and watermaze learning (Richter-Levin et al., 1995; McGahon et al., 1996). In the latter study, it was found that watermaze training occluded the increase in glutamate release normally observed after treatment of hippocampal synaptosomes with ACPD and arachidonic acid (see chapter 3.8.2). This result suggests that an increase in presynaptic inositol phospholipid metabolism may underlie the increase in glutamate release following learning. A similar occlusion of the effects of arachidonic acid and ACPD on glutamate release has been observed after LTP induction (McGahon and Lynch, 1994).

More direct support for the notion that cellular mechanisms engaged by spatial learning resemble those engaged by the induction of LTP was provided by the recent study of Richter-Levin et al. (1997). In this experiment, rats were trained in the watermaze before the induction of LTP. 2 hr after tetanization, rats were decapitated and dentate synaptosomes were prepared. Spatial training was found to occlude the increase in glutamate release normally seen after LTP induction, whereas visible platform training resulted only in a partial occlusion of the tetanus-induced increase in glutamate release. These results provide additional evidence that LTP and learning activate a common pathway in the up-regulation of glutamate release. However, such data do not constitute proof that this common pathway is involved in learning: the finding that glutamate release increases following spatial learning does not prove that the former phenomenon is necessary for the latter.

Glutamate release is not the only cellular mechanism activated by both spatial learning and LTP induction. It has been reported that a correlation exists between PKC activity and spatial learning in different strains of mice (Wehner et al., 1990). It has also been found that watermaze training in rats causes an increase in presynaptic PKC activation which occludes the increase normally induced by co-application of ACPD and arachidonic acid (McGahon et al., 1996). Whilst not providing direct proof of a link between LTP and learning, further data obtained using this approach may ultimately bring us closer to the conclusion that LTP and learning are indeed supported by similar cellular mechanisms.

2.6.5 Studies involving the use of perforant path tetanization as a CS

A number of studies have been conducted in which a perforant path tetanus served as the conditioned stimulus (CS) in an associative learning task. However, results obtained using this novel approach have proved difficult to interpret.

In one such study, perforant path tetanization was used as the CS signalling footshock in a conditioned suppression task (Laroche et al., 1989). In rats that learned the task, robust LTP developed, but both learning and LTP could be prevented by the application of NMDA receptor antagonists or by sub-threshold tetanization. A later study revealed that the forgetting of such a task assessed by retention testing paralleled the decay of LTP; i.e. a rapid decay of LTP was associated with poor retention performance. However, the induction of LTP in the perforant path may simply constitute an increase in the salience of the CS, in which case the apparent parallel between LTP and learning may simply be an artifact of the fact that the level of LTP induced and CS intensity are confounded (see Jeffery, 1997 for discussion of this issue).

However, it has also been reported that stimulation at a lower frequency normally not sufficient to result in a change in synaptic efficacy can induce an increase in EPSP slope when paired with

footshock (Matthies et al., 1986). In this experiment, a 15 Hz tetanus served as the CS in a shock-motivated shuttlebox active avoidance task. Good learners showed a greater increase in the EPSP than poor learners. However, similar criticisms apply to this study as to the correlational studies of LTP and learning discussed in section 2.6.4. In particular, the fact that poor learners necessarily received more shocks might have resulted in increased stress, a factor known to impair LTP.

Hence, studies in which the tetanus is used as a CS have so far failed to provide convincing evidence for the mechanistic equivalence of LTP and learning.

2.6.6 Pharmacological modulation of synaptic plasticity and learning

If the cellular mechanisms of LTP are identical to the cellular mechanisms of learning, an obvious prediction is that the pharmacological blockade of LTP should prevent learning. Almost every element in the LTP induction cascade has been targeted in order to test this prediction. The pharmacological modulation of mGluR function is discussed in chapter 3.12. However, the following section will concentrate only on those studies involving blockade of the NMDA receptor.

Morris et al. (1986a) provided the earliest report that the blockade of NMDA receptors could impair learning. It was found that chronic intraventricular infusion of the NMDA antagonist AP5 impaired the acquisition of a watermaze reference memory task, whilst having no effect on the performance of a visual discrimination task. These results are reminiscent of those obtained following hippocampal lesions (Morris et al., 1982, 1986b) Consistent with previous studies, chronic AP5 infusion was also found to block dentate LTP.

A subsequent study revealed that the application of AP5 after watermaze training did not affect the retrieval of previously learned spatial information (Morris, 1989). The results of this study, in conjunction with those of Morris et al. (1986a), imply that NMDA receptor activation is necessary for the acquisition, but not the subsequent recall of spatial information. Furthermore, the absence of an impairment in the expression of previous learning suggests that the acquisition deficit induced by AP5 application is unlikely to be an artifact of general sensorimotor dysfunction. In a further experiment, the effect of AP5 on spatial reversal learning was investigated. Rats were trained to a fixed platform location in the absence of any drug treatment. The animals were then divided into four groups. Two groups were implanted with osmotic minipumps containing aCSF; the other two were implanted with pumps containing 30 mM D-AP5. One AP5 group and one aCSF group were tested for their ability to locate the original platform. The other two groups were trained to locate a platform in a novel position. AP5-treated rats were unimpaired relative to controls in locating the original platform position, confirming the absence of an AP5-induced retention deficit. However, AP5 infused rats learned the novel platform position more slowly than controls (Morris, 1989). At face value,

these results provide further evidence that under closely matched experimental conditions, NMDA receptor activation is only necessary for the acquisition of new information. However, it is also possible that AP5 impaired learning of the new platform location by causing maladaptive and inflexible perseverative searching in the original platform location (see chapter 5.1.2).

The studies discussed above provide evidence that intraventricular AP5 infusion causes a selective impairment in the acquisition, but not the retrieval, of spatial information. In order to provide convincing evidence that the blockade of NMDA receptors within the hippocampus itself is responsible for the AP5-induced deficit, acute intrahippocampal infusions are preferable to chronic i.c.v. administration of the drug. Owing to its elongated structure, the hippocampus is not an ideal target for drug injection at a single site. However, fibre tracts that border the hippocampus, e.g. the alveus, help to prevent drug infusion into adjacent structures (Morris et al., 1989). Moreover, the dorsal hippocampus appears to be more important for spatial learning than the ventral portion, suggesting that it may not be necessary to attain high drug concentrations in the relatively inaccessible ventral region of the structure (Moser et al., 1993c). In order to estimate the likely spread of AP5, Morris et al. (1989) injected radiolabelled [^{3}H] D,L-AP4 and [^{3}H] D,L-AP7 directly into the hippocampus. Diffusion of these drugs was reasonably well confined to the hippocampus, with the density of the radiolabel falling sharply at the boundaries of the hippocampus. Similar results have recently been obtained with [^{3}H] D-AP5 (Steele and Morris, 1999).

Consistent with the idea that blockade of hippocampal NMDA receptors is responsible for the watermaze deficit, animals given acute intrahippocampal infusions of AP5 are similarly impaired to those given i.c.v. infusions. Morris et al. (1989) tested rats on a standard watermaze reference memory task, consisting of 8 trials per day for three days, followed 24 hr later by a transfer test with the platform absent. 15 min prior to each testing session, rats were given an acute infusion of D,L-AP5. Drug-treated rats took longer than controls to find the platform during acquisition, and performed at chance in the transfer test. Acute intrahippocampal infusion of D-AP5 has subsequently been found to impair performance in a delayed matching-to-place task in the watermaze (Steele and Morris, 1999; see below).

The selective AP5-induced block of memory formation but not expression (Morris et al., 1986a; Morris, 1989) parallels the effects of AP5 on the induction, but not the expression of LTP (Davies and Collingridge, 1989). Furthermore, it was reported that the dose response profile of the AP5-induced learning impairment parallels that of the LTP blockade *in vivo*, such that no dose of AP5 can block LTP without also affecting learning (Davis et al., 1992). Estimates of extracellular AP5 concentrations obtained by microdialysis suggested that the effective concentration of AP5 was similar to that necessary to block LTP *in vitro*.

Taken as a whole, the results described so far would seem to constitute robust evidence that hippocampal NMDA receptor-dependent synaptic plasticity is necessary for spatial learning.

However, such a view is not universally accepted. For instance, AP5 infusion is associated with sensorimotor disturbances, a finding that has led some researchers to question whether the argument for a learning impairment is valid, despite the variety of control tasks carried out (e.g. Keith and Rudy, 1990; Cain et al., 1996). This issue is discussed in some detail in chapter 5.1.

However, even if the AP5 distribution could be limited exclusively to the hippocampus, at a concentration sufficient to block LTP, and without causing sensorimotor deficits, the possibility remains that a learning impairment might result from NMDA receptor-mediated actions which are separable from the role of NMDA receptors in LTP. For instance, AP5 has been reported to decrease hippocampal theta, although this effect is only seen at high doses that cause marked sensorimotor side effects (Leung and Desborough, 1988). However, moderate doses of AP5 have been found to reduce cell excitability as indicated by the population spike of the extracellularly recorded evoked potential (Errington et al., 1987; Abraham and Mason, 1988; S. J. Martin, unpublished observations). In addition, it has been reported that hippocampal complex cell firing is impaired by NMDA receptor antagonists (Abraham and Kairiss, 1988). This raises the possibility that NMDA receptors may be involved in the routine functioning of the hippocampal circuitry, in addition to their role in synaptic plasticity (see Bekkers and Stevens, 1990b). This possibility can never be ruled out in behavioural studies involving pharmacological NMDA receptor blockade. The aim of most such studies is merely to confirm that NMDA receptor antagonists impair performance in tasks such as the watermaze by selectively disrupting learning, whilst sparing all other behavioural processes.

In addition to the critical interpretation of existing data, new experimental evidence has further complicated the story. Bannerman et al. (1995) found that when rats were given spatial pre-training in one watermaze prior to minipump implantation and postoperative spatial training in a second maze, the delivery of AP5 had almost no effect on postoperative learning. However, rats given non-spatial pre-training were still impaired when tested in the second watermaze during AP5 infusion. In contrast, rats given hippocampal lesions after training in the first watermaze were severely impaired postoperatively, regardless of the type of pre-training given. These results suggest that the activation of NMDA receptors is not necessary simply for the learning of a single platform location, once other task demands have been learned. Note that the integrity of the hippocampus is still required under these circumstances. The nature of these "other task demands" remains unclear. Saucier and Cain (1995) have reported that merely training undrugged rats on a non-spatial task results in unimpaired spatial learning in the same pool after administration of AP5. The interpretation of these data is difficult and controversial (see Morris et al., 1996). Nevertheless, it is clear that NMDA receptor activation is not, as previously thought, required for all forms of place learning.

However, spatial reference memory tasks may not provide a very sensitive index of learning ability. A more conventional way to assess memory impairments, and to dissociate memory from non-mnemonic performance factors, is to look for a delay-dependent effect of the treatment in question. Cole et al. (1993) studied the effects of the competitive NMDA receptor antagonist CPP and the non-

competitive antagonist MK-801 in an operant delayed matching-to-position task, with delays between 0 and 30 s (see Dunnett, 1985). Administration of CPP caused a delay-dependent impairment in matching-to-position performance, with no deficit at the 0 s delay. However, MK-801 produced a delay-independent impairment. These results suggest that whereas CPP genuinely impairs memory, MK-801 causes a performance deficit at all delays. Furthermore, a perseverative deficit can be ruled out in the case of CPP, since such a deficit would be expected to affect all delays equally. However, impaired performance under MK-801 was associated with an increased speed of responding, consistent with either a perseverative deficit, or a stimulant action of the drug.

Similar results have been obtained with MK-801 and the competitive NMDA receptor antagonist CGS 19755 in a spatial working memory task carried out in the 8-arm radial maze (Li et al., 1997). All arms were baited, and rats were trained to a predetermined criterion for collecting all rewards without re-visiting arms. At this point drug treatment was started. In one session, a 5 min delay was imposed between the third a fourth arm choices; in a subsequent session, no delay was introduced. Application of both drugs resulted in a deficit in the delay condition only, with no impairment in the non-delay task.

The recent development of a novel task has enabled the investigation of delay-dependent effects in the watermaze. Steele and Morris (1999) trained rats to find a novel platform position on each day of training; the platform position remained constant within each daily session. Hence the performance on trial 2 of each day constitutes a matching-to-place task, in which the rat must remember the location of the platform presented on trial 1 after variable delays. It was found that rats infused with D-AP5 via chronic i.c.v. minipumps were unimpaired at the shortest delay of 15 s between trials 1 and 2. However, if 20 min or 2 hr were allowed to intervene, AP5-treated rats were amnesic. This result is hard to interpret as anything other than a memory impairment, although one possible criticism of both the above study and that conducted by Cole and colleagues concerns the treatment of animals during the delay period. Cole et al. (1993) returned the rats to their home cages during the 5 min delay period, whereas no equivalent manipulation occurred in the non-delay period. In an attempt to control for such factors, Steele and Morris (1999) returned rats to their home cages after trial 1 at all delays. However, the cages were returned to the animal room during the 20 min and 2 hr delay periods, but obviously not during the 15 s delay. This change in context between different delays might account for an apparent delay-dependent effect. However, a subsequent study was carried out using 15 s and 2 hr delays only. During each of these delay periods, rats remained in the watermaze room. Under these circumstances, a delay-dependent effect was still observed, ruling out the possibility that the result might be an artifact of a change in context between delays (Steele and Morris, 1999).

It is not immediately obvious how these results can be reconciled with the findings of the two-pool study carried out by Bannerman et al. (1995). In the matching-to-place study, rats received extensive spatial pretraining in the same pool in which they were tested during AP5 infusion. However, a deficit in matching-to-place performance was still observed, despite the fact that, before minipump

implantation, rats had clearly learned all aspects of the task except the daily requirement to remember a novel location. This result apparently contradicts that of the two-pool study in which spatial pre-training in one environment was sufficient to allow NMDA receptor-independent place learning in a novel environment. The reason for this apparent discrepancy is unclear, although the demands of the two tasks are very different. For instance, in the matching-to-place task, single trial leaning of a novel location is required on each day, compared to the one trial per day learning of a fixed location required in the postoperative phase of the two pool study. It is possible that the former task makes considerably greater demands on hippocampal synaptic plasticity than the latter task, which simply requires the gradual cumulative learning of a single platform location.

Despite this apparent discrepancy, the results of Steele and Morris (1999) provide strong evidence that NMDA receptor activation is necessary for the formation of spatial memories, at least under some circumstances. Furthermore, it is very unlikely that non-mnemonic factors such as sensorimotor or motivational effects of AP5 infusion can account for the delay-dependent deficit observed. As mentioned above, a role of NMDA receptors in learning does not necessarily imply that synaptic plasticity is involved in learning. The inference is merely correlational. However, whilst not proving the hypothesis, the finding that NMDA receptor antagonists cause a delay-dependent deficit in spatial learning provides some of the strongest current evidence suggesting a link between hippocampal synaptic plasticity and learning.

2.6.7 Targeted disruption of genes coding for proteins involved in the induction or expression of LTP

Recent advances in transgenic technology have provided the possibility of targeted gene disruption as an alternative to pharmacological intervention in studies of LTP and learning. For instance, mice lacking the α subunit of CaMKII (see section 2.4.5) showed a spatial learning deficit in the watermaze, and a deficit in LTP *in vitro* (Silva et al., 1992a, 1992b). Normal synaptic transmission was unaffected, and although mutant mice were initially impaired on a visible platform task, they eventually caught up with wild-type animals by the fifth block of training trials. Similar behavioural and electrophysiological results have been obtained with mice deficient in the *fyn* gene which codes for a non-receptor tyrosine kinase (Grant et al., 1992).

A large number of gene knockout mice have now been produced, and many have undergone electrophysiological and behavioural testing. For instance, mice deficient in the γ subunit of PKC show a very mild impairment in spatial learning, but show a profound impairment in CA1 LTP (Abeliovich et al., 1993a and b). However, further investigation revealed that if preceded by the delivery of priming stimulation consisting of 900 pulses at 1 Hz, normal LTP was obtained following tetanization. The mechanism of this priming effect is unknown. Similarly, mice deficient *in Thy-1*, a neuronal cell adhesion molecule, are unimpaired in the watermaze, but are severely deficient in

dentate LTP *in vivo* under anaesthetic (Nosten-Bertrand et al., 1996). However, it was found that local disinhibition restored the capacity for normal LTP. A subsequent study revealed that dentate LTP, although still impaired, could be obtained without disinhibition in freely moving *Thy-1* mutant mice (Errington et al., 1997). These results illustrate a serious difficulty in drawing parallels between LTP and learning after an experimental manipulation. If a treatment results in a block of LTP, there is always a possibility that under another set of circumstances such a block might not be obtained. The converse is, of course, also true (see chapter 3.10.5). Hence, the question of whether synaptic plasticity is available to a behaving animal may be very difficult to answer on the basis of conventional LTP experiments.

In addition to the general problem outlined above, there are a number of problems specific to the use of genetic techniques. For instance, none of the mutations described above is restricted to the hippocampus, and all are present throughout development. Hence, the possibility that learning impairments, if found, are not hippocampal in origin cannot be ruled out. For instance, it has been reported that *fyn* mutant mice tend to spend much of their time floating in the watermaze without swimming, a behaviour which increases escape latencies. However, lightly touching the hind feet of the mutants at the start of a trial resulted in improved swimming, and normal spatial learning (Huerta et al., 1996; note, however, that this study was based on a sample of only 6 mice per group).

An additional complication concerns the possibility that the absence of a gene product throughout development may result in unpredictable structural and functional changes in the brain. For instance, *fyn* mutant mice have an increased number of dentate granule cells and CA3 pyramidal cells, resulting in abnormalities in hippocampal morphology, the functional significance of which is unknown.

It is possible that abnormalities in transgenic animals may result either from the absence of some function performed by the normal gene product during development, or from compensatory changes occurring in response to the absence of this protein. With respect to the latter possibility, it is likely that during development in the absence of a gene coding for a specific protein, upregulation of genes coding for related proteins or alternative isoforms may occur. It has often been remarked that the deficits resulting from gene knockouts are surprisingly mild considering the apparent importance of the gene deleted. For instance, the CaMKII protein constitutes approximately 2 % of the total hippocampal protein context, yet the effects of its deletion are comparatively subtle. It is possible that compensatory changes of the sort proposed may explain this finding. If such compensatory changes are routinely induced by gene knockouts, the absence of a deficit cannot be taken as conclusive evidence that the deleted gene normally plays no role in the function investigated. Conversely, the presence of an abnormality in a mutant may reflect the activation of compensatory mechanisms, rather than the absence of the gene under investigation. For a discussion of such issues, together with the addition problem of genetic background, see Gerlai (1996a and b) and Routtenberg (1995).

Recent technological advances have overcome the problem of developmental defects to some extent. A transgenic mouse has recently been developed in which the αCaMKII gene is mutated resulting in a Ca^{2+}-independent CaMKII enzyme. Expression of this mutation results in a systematic shift in the relationship between tetanus frequency and LTD / LTP, such that LTP is deficient at low frequencies (Mayford et al., 1995). A further transgenic strain was developed in which expression of the CaMKII transgene could be temporarily suppressed. These mice exhibited deficient LTP at low tetanus frequencies, and were impaired in spatial learning. However, these deficits were reversed after the transgene was turned off (Mayford et al., 1996). These results suggest that the abnormalities in hippocampal function do not result from developmental disturbances caused by the mutation. Subsequent experiments have revealed that these activated CaMKII mice have less well-defined CA1 place fields, with lower peak firing rats than wild-type animals, a fact which might contribute to the spatial learning deficit (Rotenberg et al., 1996).

By combining the *Cre-loxP* recombination system with embryonic stem cell targeting technology, a knockout mouse has recently been created in which the gene coding for the NMDAR1 subunit is selectively deleted in CA1 pyramidal cells and only from the third week of postnatal life onwards, thus avoiding most potential developmental changes (Tsien et al., 1996a). These mice lack an NMDA receptor-mediated EPSC and do not exhibit CA1 LTP or LTD, although dentate LTP is unimpaired. Furthermore, mutant mice fail to show a bias towards the training quadrant in a watermaze transfer test following reference memory training (Tsien et al., 1996b). A subsequent study revealed that CA1 place fields in these mice were larger than those in controls and tended to have multiple peaks. In addition, cells with overlapping place fields failed to show normal temporal firing correlations (McHugh et al., 1996).

To conclude, studies involving the disruption of genes thought to be involved in synaptic plasticity have generally provided ambiguous evidence about the role of synaptic plasticity in learning, and in some cases have highlighted methodological difficulties in demonstrating such a relationship. However, the so-called "second generation" knockouts of Mayford et al. (1996) and Tsien et al. (1996a) offer a glimpse of the possibilities of this new technology. The arrival of fully inducible and regionally specific gene knockouts is likely to provide an extremely powerful tool for future studies of LTP and learning.

2.6.8 Summary

Despite all the evidence presented in this section, it is still impossible to provide a definitive answer to the question of whether the processes engaged by the induction of LTP are the same as those involved in learning. While pharmacological and gene targeting studies have provided fairly convincing evidence that elements of the cellular machinery, such as NMDA receptors, are necessary

for both LTP and learning, the problem remains that such evidence is merely correlational, and does not imply a causal relationship between the mechanisms of LTP and those of learning. A similar problem applies to demonstrations that the level of LTP that can be induced in an animal correlates with its learning ability.

The observation of LTP-like changes in synaptic efficacy during learning would constitute stronger proof of a causal relationship. However, attempts to identify learning-related increases in fEPSPs have met with little success, and may be doomed to failure on *a priori* grounds. The possibility of identifying LTP between pairs of individual neurons *in vivo* with unit recording techniques, whilst technologically daunting, may provide one way forward. Alternatively, it may be possible to devise an experimental manipulation that selectively targets recently potentiated synapses, then test the effects of this manipulation on memory recall (see chapter 8). At present, however, despite its theoretical plausibility, the hypothesis that memories are stored as changes in synaptic strength has proved impossible either to confirm or to refute.

Chapter Three

Metabotropic glutamate receptors (mGluRs): pharmacology, physiology and function

3.1 Introduction

The role of the NMDA receptor in hippocampal synaptic plasticity and spatial learning was discussed in the previous chapter (see sections 2.4.4 and 2.6.6). However, much of the experimental work described in this thesis concerns the role of the more recently identified metabotropic glutamate receptor (mGluR).

The experiments described in chapter 6 represent an attempt to characterize, in our hands, the effects of mGluR blockade on performance in a standard behavioural paradigm, the watermaze reference memory task. The effects of mGluR antagonism on LTP are then assessed in chapter 7. The mGluR antagonist used throughout, (*R*,*S*)-MCPG, is not ideal for reasons discussed in sections 3.6.3, 3.10.6, and 3.10.7. However, few other selective mGluR antagonists were available at the time when the experimental work was started, and a small literature concerning the effects of this drug on spatial learning tasks other than the watermaze already existed. In addition, the use of MCPG was considered preferable to newer drugs with higher apparent subtype specificity and potency in expression systems, but whose pharmacological properties *in vivo* were even less well characterized than those of MCPG.

The following chapter provides an overview of the role of mGluRs in hippocampal functioning, synaptic plasticity and learning. Basic aspects of mGluR physiology and pharmacology are introduced in sections 3.2-3.8 of this chapter. The remaining sections cover aspects of the literature which are more directly related to the experimental work described later in this thesis. For instance, section 3.9 on the role of mGluR activation in various forms of synchronous hippocampal activity, and section 3.11 on slow-onset potentiation, have been included to provide a background to the work with the mGluR agonist (1*S*,3*R*)-ACPD in chapter 7.5. The sections on mGluRs and synaptic plasticity and learning (3.10 and 3.12) are directly relevant to work carried out in chapters 6 and 7. Particular emphasis is placed on studies involving the mGluR antagonist, (*R*,*S*)-MCPG, a drug used throughout a large part of this thesis.

3.2 The discovery of mGluRs

Fast excitatory synapses in the mammalian central nervous system use L-glutamate as a neurotransmitter. The receptor-mediated actions of glutamate were until recently thought to be confined to the direct gating of cation channels, although glutamatergic transmission was known to be modulated by the release of neurotransmitters such as dopamine, serotonin, acetylcholine, and noradrenaline from extrinsic afferent projections. These compounds exert their effects by activating G-protein-coupled receptors linked to intracellular second messenger cascades. However, Sladeczek et al. (1985) discovered that glutamate could itself stimulate inositol 1,4,5-trisphosphate (IP3) formation in cultured striatal neurons. A similar glutamate-mediated stimulation of IP3 synthesis was subsequently demonstrated in the Purkinje cells of the cerebellum (Nicoletti et al., 1986a) and many other parts of the brain, including the hippocampus (Nicoletti et al., 1986b). Experiments in which Xenopus oocytes were injected with rat brain mRNA confirmed that this effect is dependent upon the direct activation of an expressed receptor, and not simply a consequence of membrane depolarization or calcium influx (Sugiyama et al., 1987). Activation of the glutamate receptor in these oocytes induced a calcium-dependent chloride current. This is known to be characteristic of G-protein-linked receptors which couple to phospholipase C and cause the breakdown of phosphoinositides, resulting in the formation of the protein kinase C activator diacylglycerol (DAG), and IP3 which promotes the release of intracellular calcium. This new class of G-protein-coupled glutamate receptor became known as the "metabotropic glutamate receptor" or mGluR.

3.3 Cloning of the mGluR receptor family

The mGluR family consists of a number of subtypes, 8 of which have been cloned to date (Nakanishi, 1992; Pin and Duvoisin, 1995). The first mGluR, now known as mGluR 1a (or α), was independently cloned by two groups (Masu et al., 1991; Houamed et al., 1991). The deduced amino acid sequence revealed a receptor much larger than previously identified G-protein-coupled receptors, and sharing little sequence homology with them. Seven other genes encoding mGluRs have since been identified, together with a number of splice variants. (Note that the splice variants of all mGluR subtypes are hereafter identified by Roman characters only, despite the common practice of using Greek letters to identify splice variants of mGluR1.) In common with other G-protein-coupled receptors, mGluRs consist of 7 transmembrane domains. However, ligand binding does not occur in this region, but on an uncharacteristically large N-terminal extracellular domain (Takahashi et al., 1993).

mGluRs can be classified into 3 groups according to their amino acid sequence homology. Group 1 comprises mGluRs 1 and 5, group 2 comprises mGluRs 2 and 3, and group 3 comprises mGluRs 4, 6, 7 and 8. Within group sequence identity is roughly 70 %, whilst between group identity is about 45 % (Pin and Duvoisin, 1995). The mGluRs have little sequence homology with most other G-protein-coupled receptors, although they do show significant homology with the $GABA_B$ receptor (Kaupmann et al., 1997) and a parathyroid Ca^{2+}-sensing receptor (Garret et al., 1995).

3.4 Regional and sub-cellular distribution of mGluRs

Metabotropic glutamate receptors are heterogeneously distributed throughout the brain. *In situ* hybridization and immunolabelling studies have revealed that the various mGluR subtypes show distinct but overlapping profiles of regional and sub-synaptic localization, consistent with the different roles which members of the mGluR family are believed to play. The approximate sub-cellular localization of the three mGluR groups is illustrated in figure 3.5.

3.4.1 Group I mGluRs

In adult rats, the mRNA for mGluR1 is most prominently expressed in cerebellar Purkinje cells. However, while numerous moderately labelled cells are found in the dentate gyrus, expression in CA1 is weak (Masu et al., 1991; Shigemoto et al., 1992). Similar results have been obtained by immunoblotting with an anti-mGluR1a antibody (Martin et al., 1992). Hippocampal labelling was localized to non-pyramidal cells, particularly those in the polymorph layer of the dentate gyrus, and the stratum oriens of CA1. Subsequent studies have confirmed that mGluR1a immunoreactivity is largely confined to somatostatin-containing GABAergic interneurons (Baude et al., 1993; Luján et al., 1996).

However, the alternative splice variants mGluR1b and / or c have been detected on principal cells of the hippocampus: the pattern of immunoreactivity for an antibody which binds to all three splice variants included pyramidal cells of CA3 and dentate granule cells, in contrast to mGluR1a labelling which was confined to interneurons. CA1 pyramidal cells were not labelled, even by the pan mGluR1 antibody (Luján et al., 1996).

In situ hybridization with an mGluR5 mRNA-specific probe has revealed high expression in many brain areas, including pyramidal cells throughout Ammon's horn, and dentate granule cells. Hence, group I mGluR actions in CA1 are likely to be mediated by mGluR5, rather than mGluR1, which is largely absent. Immunohistochemical studies have confirmed this pattern of distribution (Shigemoto et al., 1993). Overall levels of mGluR5 receptor protein correlate well with developmental changes in agonist-stimulated PI turnover in brain slices, suggesting that this receptor mediates the major component of the phosphoinositide response (Casabona et al., 1997b).

Electron microscopic examination of immunogold-labelled hippocampal slices has revealed that group I mGluRs are localized postsynaptically on dendritic spines. mGluRs 1 and 5 are concentrated at perisynaptic sites, adjacent to the postsynaptic specialization (Baude et al., 1993; Nusser et al., 1994; Luján et al., 1996). However, some pre-synaptic immunoreactivity for mGluR5 was reported

by one group (Romano et al., 1995). Biochemical evidence also suggests the existence of a presynaptic group I-like mGluR (Herrero et al., 1992; see section 3.8.2)

3.4.2 Group II mGluRs

mGluR2 is most heavily expressed in the Golgi cells of the cerebellar cortex. Within the hippocampus, mGluR2 mRNA is prominent in the dentate gyrus, but is absent in Ammon's horn. Pyramidal cells of the entorhinal cortex are also heavily labelled (Ohishi et al., 1993a; Fotuhi et al., 1994). Immunoreactivity to the mGluR2 receptor protein is localized to perforant path fibres from the entorhinal cortex to the stratum lacunosum-moleculare of the dentate gyrus and CA1-CA3, and the mossy fibres from dentate granule cells to the stratum lucidum of CA3, consistent with the suggested role of mGluR2 as a presynaptic autoreceptor in these pathways (Neki et al., 1996; Shigemoto et al., 1997).

In situ hybridization signals for mGluR 3 have been detected not only in neurons, but also in many glial cells throughout the brain. In the hippocampus, dentate granule cells are weakly labelled, but mGluR3 is absent in Ammon's horn; moderate to weak expression is also seen in the entorhinal cortex (Ohishi et al., 1993b; Tanabe et al., 1993).

Curiously, electron microscopic examination of group II mGluR immunogold-labelled sections reveals an extrasynaptic concentration of reactivity, with little labelling within the pre-synaptic membrane specialization, suggesting that group II mGluRs may only be activated after the accumulation of glutamate during, for instance, repeated stimulation of afferents (Shigemoto et al., 1997).

3.4.3 Group III mGluRs

Of the four members of group III, only mGluRs 4, 7, and 8 are expressed in the hippocampus: mGluR 6 is preferentially localized to retinal ON bipolar cells (Masu et al., 1995). mGluR4 is most highly expressed in cerebellar granule cells, but weak expression has been reported in a number of hippocampal areas (Kristensen et al., 1993; Tanabe et al., 1993; Ohishi et al., 1995). Immunolabelling for mGluR4a is generally weak in the hippocampus, but prominent in the inner third of the dentate molecular layer, corresponding to the termination zone of the associational / commissural fibres, suggesting an autoreceptor role for mGluR4a in this pathway (Shigemoto et al., 1997). A second splice variant, mGluR4b, has recently been characterized, but its pattern of expression is currently unknown (Thomsen et al., 1997).

In contrast to mGluR4, prominent expression of mGluR7 has been reported in dentate granule cells

and in the pyramidal cell layer throughout Ammon's horn (Ohishi et al., 1995; Kinzie et al., 1995). Two splice variants of mGluR7 have been identified. Immunolabelling has revealed that mGluR7a is prominent on interneurons in the stratum oriens, radiatum and lacunosum–moleculare of both CA1 and CA3. No labelling is observed on pyramidal cells. In the dentate gyrus, labelling is intense in the middle third of the molecular layer, corresponding to the termination zone of the medial perforant path, consistent with an autoreceptor role for mGluR7a in this pathway.

Expression of mGluR8 mRNA is weak in the hippocampus (Duvoisin et al., 1995; Saugstad et al., 1997). However, prominent immunolabelling of mGluR8 protein has been reported in the outer third of the dentate molecular layer, and the superficial portion of the CA3 stratum lacunosum moleculare, both of which correspond to terminal zones of the lateral perforant path, suggesting an autoreceptor role for mGluR8 (Shigemoto et al., 1997).

In contrast to group II mGluRs, group III immunolabelling is concentrated at axon terminals, within the presynaptic membrane specialization (Shigemoto et al., 1997).

3.5 Transduction mechanisms of mGluRs

The classification of mGluRs based on sequence homology is supported by the transduction mechanisms of the individual subtypes. Figure 3.5 shows a schematic view of the transduction mechanisms of the three mGluR sub-groups at a generic hippocampal synapse.

3.5.1 Group I mGluRs

Studies of mGluRs expressed in various cell lines have consistently revealed that group I mGluRs stimulate phospolipase C and phosphoinositide hydrolysis. Similar results have been obtained in native systems (for review, see Sudzak et al., 1994; Pin and Duvoisin, 1995; Conn and Pin, 1997). Activation of group I mGluRs has also been reported to stimulate cAMP formation in expression systems (Aramori et al., 1992; Pickering et al., 1993; Joly et al., 1995), although it is not known whether this coupling is present in real neurons, where levels of mGluR expression are lower than those obtained in artificial expression systems. Nevertheless, co-activation of group I and II mGluRs causes a synergistic potentiation of the cAMP response to agonists of G_s-coupled receptors in neonatal rat hippocampal slices (Schoepp et al., 1996). However, this effect is mediated by the activation of group II mGluRs in adult slices; activation of group I and III mGluRs is ineffective (Winder and Conn, 1995).

3.5.2 Group II mGluRs

Group II mGluRs inhibit forskolin-stimulated cAMP formation in mammalian cell lines. This effect is sensitive to pertussis toxin treatment, indicating that the G_i type of G-protein is involved (Tanabe et al., 1992; Tanabe et al, 1993). Similar results have been obtained in native systems, such as cell cultures from various brain regions (Prezeau et al., 1994), and hippocampal slices (Wright and Schoepp, 1996). However, it is possible that the effect of group II mGluR activation on cAMP accumulation in response to the adenylate cyclase activator forskolin does not provide an accurate index of the response under normal circumstances. As mentioned above, group II mGluRs can potentiate increases in cAMP formation in response to agonists of G_s-coupled receptors (Winder and Conn, 1995). Hence, the natural physiological response to group II mGluR activation may be opposite to that assayed in the presence of forskolin.

3.5.3 Group III mGluRs

Group III mGluRs are also negatively coupled to adenylate cyclase, although more weakly than group II. However, this may not be the preferred coupling of group III mGluRs in their native environments (see Pin and Duvoisin, 1995; Conn and Pin, 1997 for reviews). In contrast to the findings described above for group II mGluRs, the selective group III agonists L-AP4 and L-SOP have been found to inhibit G_s-coupled receptor agonist-induced increases in cAMP formation in hippocampal slices, in addition to inhibiting the increase caused by the application of forskolin (Wright and Schoepp, 1996).

3.5.4 mGluRs coupled to phospholipase D

There is evidence that some mGluRs may be coupled to the activation of phospholipase D (PLD) (Boss et al., 1994; Holler et al., 1993). The pharmacological profile of this response is not consistent with that of any known mGluR subtype, and may therefore represent a novel, currently uncloned receptor (Albani-Torregrossa et al., 1998).

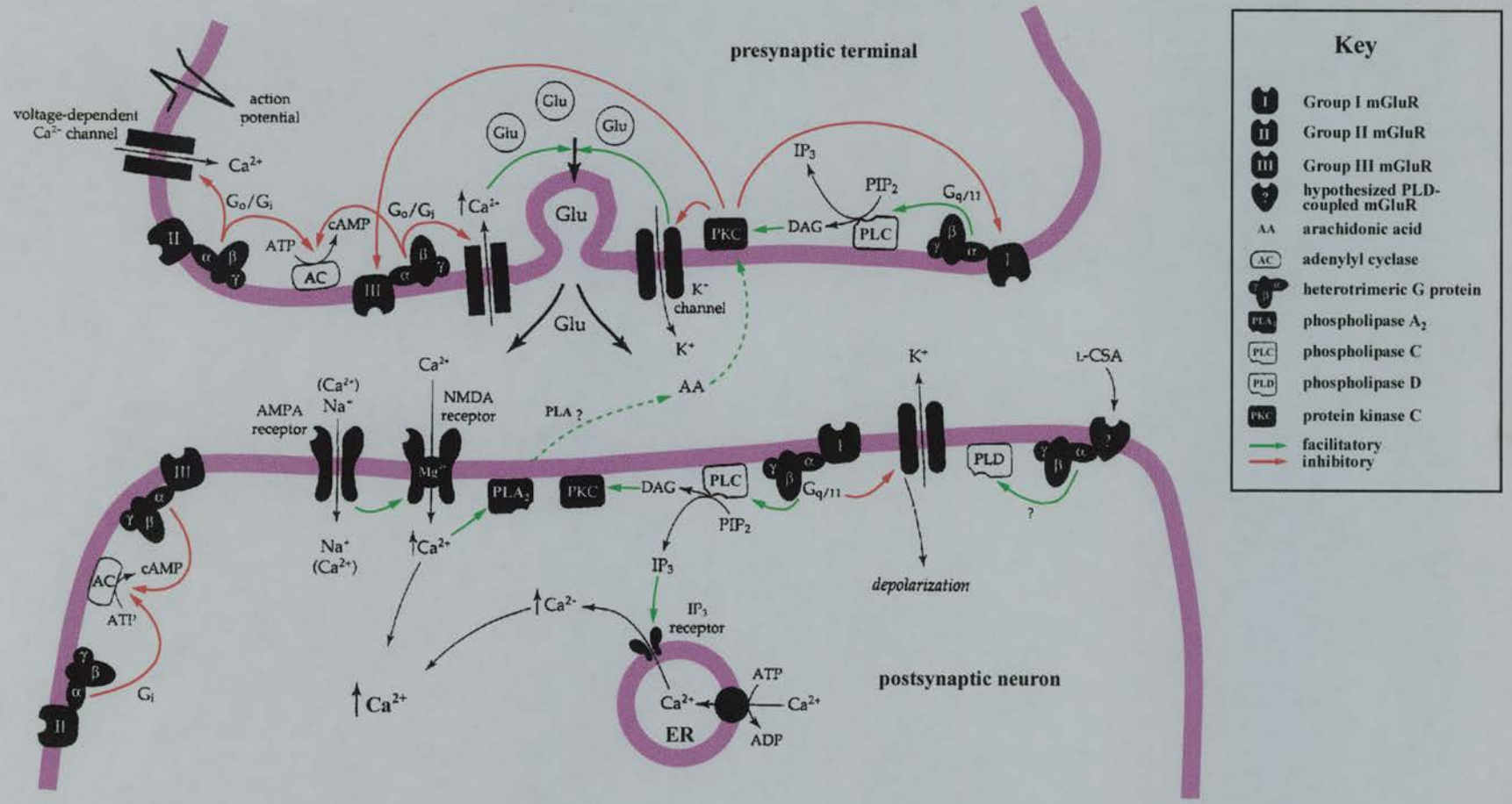


Fig. 3.5 Schematic diagram of the second messenger coupling of mGluRs. (modified from Toms and Roberts, 1997).

3.6 mGluR pharmacology

3.6.1 mGluR agonists

Pharmacological studies of the role of mGluRs have consistently been hampered by the lack of selective agonists and antagonists. Whilst AMPA and NMDA are inactive at mGluRs, ibotenate (an NMDA receptor agonist) and quisqualate (an AMPA / kainate receptor agonist) are effective mGluR agonists, although their actions at ionotropic glutamate receptors make them unsuitable for use in native systems. However, numerous mGluR-selective agonists have been developed in the last few years.

The earliest selective mGluR agonist was a conformationally restricted form of glutamate, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*trans*-ACPD; but see Schoepp et al. (1991b) for discussion of nomenclature), a racemic mixture of (1*R*,3*S*)- and (1*S*,3*R*)-enantiomers, the latter of which is now known to be the active form (Palmer et al., 1989; Irving et al., 1990). In heterologous expression systems, (1*S*,3*R*)-ACPD activates both group I and group II receptors, but has higher affinity for the latter group (Saugstad et al., 1995 see table 3.6.2). Many more mGluR agonists have since been synthesized, several of which are listed in table 3.6.1

3.6.2 mGluR antagonists

Early studies suggested that D,L-AP4 could antagonize the agonist-induced increase in phosphoinositide hydrolysis in adult hippocampal slices (Nicoletti et al., 1986a), an action mediated by the L-isomer of the drug (Schoepp and Johnson, 1988). However, L-AP4 is at best a weak antagonist of PI hydrolysis, and has been found to be inactive in many preparations, such as slices from immature rats (Nicoletti et al., 1986b), and in cultured neurons (e.g. Patel et al., 1990). Moreover, L-AP4 is inactive at group I PI-coupled mGluRs, although it has been found to be a selective agonist at group III mGluRs, as discussed above. The reason for the reported effects of L-AP4 on PI hydrolysis are unknown, although cross-talk between receptor subtypes, or the existence of an unknown AP4 sensitive PI-coupled mGluR may explain these findings. Nevertheless, L-AP4 is clearly an unsuitable antagonist for use in physiological studies of mGluR function.

Another putative mGluR antagonist used in many early studies is L-amino-3-phosphonopropionate (L-AP3). This compound was found to inhibit mGluR agonist-induced effects, including the increase in PI hydrolysis, in many different preparations (Schoepp et al., 1991b). However, L-AP3 does not antagonize the depolarization and inhibition of slow afterhyperpolarization induced by *trans*-ACPD application in CA1 pyramidal cells (Stratton et al., 1990). A similar failure to antagonize the ACPD-

induced depolarization in rat thalamic neurons has been reported (Salt and Eaton, 1991). These findings, together with the low potency and non-competitive action of L-AP3, limit its usefulness as an mGluR antagonist.

More recently, a family of phenylglycine derivatives has been described, some exhibiting antagonist, and others agonist properties at mGluRs, such as (*S*)-DHPG mentioned above (see Watkins and Collingridge, 1994 for review). (*S*)-4-carboxyphenylglycine ((*S*)-4CPG) and the racemic form of its α-methyl derivative (*R,S*)-α-methyl-4-carboxyphenylglycine ((*R,S*)-MCPG) were reported to block (1*S*,3*R*)-ACPD stimulated PI hydrolysis in rat cerebral cortical slices. In contrast to L-AP3, these antagonists were found to inhibit the ACPD-induced depolarization in rat thalamic neurons and neonatal rat motor neurons (Eaton et al, 1993). The (+)- or (*S*)-isomer of MCPG has since been identified as the active form (see table 3.2). Since the discovery of the properties of phenylglycines, several more selective mGluR antagonists have been characterized. A range of commonly used mGluR antagonists are listed in table 3.6.1

Table 3.6.1 mGluR agonists and antagonists (data taken from Jane et al., 1994, 1995; Pin and Duvoisin, 1995; Pelliciari et al., 1995; Gomeza et al., 1996; Toms et al., 1996; Conn and Pin, 1997; Roberts, 1998). The results are based mainly on biochemical measurements, e.g. phosphoinositide hydrolysis and cAMP formation, in heterologous expression systems. Full drug names are included in the list of abbreviations on page xvii.

	Group I	Group II	Group III
Subtypes	mGluR1 mGluR5	mGluR2, mGluR3	mGluR4 mGluR6 mGluR7 mGluR8
Principal agonists (in order of potency)	Quisqualate > (*S*)-DHPG[a] ≈ L-glutamate > (1*S*,3*R*)-ACPD > L-CCG-I > (*S*)-3-HPG[a] > *t*-ADA	LY354740[a] > DCG-IV[b] > L-CCG-I > (2*R*,4*R*)-APDC[a] > L-glutamate > (1*S*,3*S*)-ACPD ≈ (1*S*,3*R*)-ACPD > (*S*)-4C3HPG[c] > ibotenate	L-AP4[a] > L-SOP[a] > L-glutamate > L-CCG-I > (1*S*,3*S*)-ACPD ≫ (1*S*,3*R*)-ACPD
Principal antagonists (in approximate order of potency)	AIDA[a] > (*S*)-4CPG ≥ (S)-4C3HPG[c] ≥ (+)-MCPG	PCCG-IV > MCCG-I[a] > MPPG > MSPG > MTPG > (+)-MCPG	(*R*,*S*)-CPPG[a] > MPPG > MSOP[a] > MAP4

[a] These compounds are believed to be selective agonists or antagonists for the particular mGluR group in which they are listed.

[b] This compound also acts as an NMDA receptor agonist.

[c] This compound is both a group I mGluR antagonist, and a group II agonist.

3.6.3 The pharmacological properties of MCPG

Unlike (*S*)-4CPG, which has agonist properties at group II mGluRs, MCPG antagonizes both group I and group II mGluRs in cellular expression systems (Hayashi et al., 1994; Thomsen et al., 1994). However, it has been reported that (+)-MCPG does not inhibit the ACPD-induced reduction in forskolin-stimulated cAMP accumulation in adult hippocampal slices, despite inhibiting ACPD-induced PI hydrolysis in a competitive manner, suggesting a functional selectivity for group I mGluRs (Riedel et al., 1995a). Nevertheless, MCPG has been found to inhibit the ACPD-induced reduction in forskolin-stimulated cAMP accumulation in guinea pig cortical slices (Kemp et al, 1994), as well as antagonizing a number of group II-mediated electrophysiological responses in the hippocampus, an issue which will be discussed later. In addition, recent studies have revealed that (+)-MCPG acts as an agonist at a group II-like receptor (or receptors) in slices taken from hippocampal area CA1 (Breakwell et al., 1998), or basolateral amygdala (Keele et al., 1995). The recent finding that (+)-MCPG can stimulate phospolipase D activation in the hippocampus raises the possibility that it acts as an agonist at an unidentified mGluR subtype (Pelligrini-Giampietro et al., 1996).

One further complication concerning the use of MCPG as an antagonist of PI-coupled mGluRs concerns the fact that MCPG antagonizes the *trans*-ACPD-induced increase in PI turnover far more potently than the increase in PI turnover induced by glutamate application in both hippocampal and cerebellar slices (Littman and Robinson, 1994). Similar results have recently been described in rat visual cortical slices (Huber et al., 1998). These findings are in agreement with data from non-neuronal cells expressing either mGluR1a or mGluR5a. (+)-MCPG was found to potently inhibit the effect of (1*S*,3*R*)-ACPD on both subtypes. However, (+)-MCPG exhibited lower potency as an antagonist of glutamate-mediated effects on mGluR1a, and was almost completely ineffective against the actions of glutamate on mGluR5a (Joly et al., 1995; Brabet et al, 1995). On the basis of these results, Brabet et al. (1995) suggested that glutamate and (1*S*,3*R*)-ACPD bind to different sites on the mGluR receptor. The high expression of mGluR5 within the hippocampus and cortex would explain the failure of MCPG to block glutamate-stimulated PI hydrolysis in these regions. Alternatively, it is possible that much of the response to glutamate is mediated by unidentified ACPD-insensitive mGluR subtypes. These results imply that the mere demonstration of an MCPG-induced inhibition of the effects of ACPD application cannot be taken as conclusive evidence that a physiologically relevant blockade of mGluRs has been achieved.

Despite these potential problems, MCPG was the best mGluR antagonist available at the time when the work described in this thesis was started. The main drugs used in chapters 6 and 7 are (*R*,*S*)-MCPG and (1*S*,3*R*)-ACPD. (-)-MCPG was used on one occasion as a control. A detailed pharmacological characterization of (-) and (+)-MCPG, and (1*S*,3*R*)-ACPD is presented in table 3.6.2.

Table 3.6.2 Potency (EC_{50}, IC_{50}, or K_b values in μM) of MCPG and ACPD determined in cell lines expressing a particular mGluR subtype (values taken from Conn and Pin, 1997; Saugstad et al., 1997; Hayashi et al., 1994), or in the case of ionotropic receptors, from cultured striatal neurons (ACPD: Manzoni et al., 1990), and cerebral cortical membranes (MCPG: Thomsen et al., 1994).

	(-)-MCPG[a]	(+)-MCPG	(1S,3R)-ACPD
AMPA receptor	—	> 1000	> 1000[b]
NMDA receptor	—	> 1000	> 1000[b]
mGluR1a	n.e.	40-200	10-80
mGluR5a	—	>200	5-7
mGluR2	n.e	100-1000	18
mGluR3	—	>1000	8
mGluR4a	n.e.	n.e.	>>300
mGluR6	—	—	300
mGluR7	—	n.e.	—
mGluR8	—	320[c]	47

n.e. no effect

— not tested

[a] (-)-MCPG has also been reported to be ineffective in antagonizing the ACPD-induced depolarization of neonatal rat motoneurones and the excitation of thalamic neurons (Jane et al., 1993), as well as the ACPD induced increase in phosphoinositide metabolism in cortical slices (Eaton et al., 1993). It has also been found to be ineffective in blocking LTP (Riedel et al., 1995).

[b] *trans*-ACPD was used in these cases.

[c] The racemic form, (*R,S*)-MCPG was used in this case.

3.7 Modulation of ion channels and cell excitability

3.7.1 Modulation of potassium channels

Metabotropic glutamate receptors are involved in the modulation of several potassium conductances, with consequent effects on cell excitability (see figure 3.5). Early studies indicated that mGluR activation could induce a slow membrane depolarization of hippocampal CA1 neurons, associated with an increase in membrane resistance; stimulation of mGluRs was also reported to block the slow after-hyperpolarization current (I_{AHP}) in CA1 pyramidal cells, a phenomenon known to be mediated by Ca^{2+}-activated potassium channels (Stratton et al., 1989; Charpak et al., 1990; Baskys et al., 1990). It has been reported that application of mGluR agonists causes an inhibition of spike frequency adaptation in hippocampal neurons, an effect consistent with an mGluR-mediated blockade of I_{AHP} (Stratton et al., 1989; Desai and Conn, 1991). Activation of mGluRs in hippocampal pyramidal cells has also been reported to block several other potassium currents, such as a leak potassium conductance (I_{leak}), active at the resting membrane potential (Guérineau et al., 1994), a slow, non-inactivating, voltage-dependent potassium conductance (I_M) (Charpak et al., 1990), and a slowly inactivating voltage-dependent current ($I_{K(slow)}$) (Lüthi et al., 1996). The inhibition of these potassium currents is thought to underlie the slow depolarization and increase in membrane resistance associated with mGluR activation.

Pharmacological studies suggest that the postsynaptic depolarization and blockade of spike frequency adaptation are mediated by the activation of group I mGluRs. Davies et al. (1995) found that these electrophysiological effects could be elicited in CA1 pyramidal neurons by the selective group I agonist (*S*)-DHPG. In contrast, DCG-IV and L-AP4, agonists at group II and III mGluRs respectively, had no effect on the passive membrane properties, or spike frequency adaptation. Furthermore, both (+)-MCPG (a group I and II antagonist) and (*S*)-4CPG (a group I antagonist) inhibited the postsynaptic actions of (1*S*,3*R*)-ACPD. (+)-MCPG was also found to antagonize the actions of (*S*,*R*)-DHPG. (The effects of (*S*)-4CPG were not tested.) Since (1*S*,3*R*)-ACPD elicits normal postsynaptic excitatory effects in mGluR1 knockout mice (Aiba et al., 1994; Conquet et al., 1994), Davies et al. (1995) tentatively suggest that these effects are most likely to be mediated by the mGluR5 subtype of group I, although a role of mGluR1 in normal animals cannot be excluded.

3.7.2 Activation of non-specific cation currents

Stimulation of postsynaptic mGluRs can also increase cell excitability by the activation of non-specific cation currents. These currents can be generated in a number of ways, such as activation of a Na^+ / Ca^{2+} exchanger (McBain et al., 1994), a calcium independent, non-specific cation channel

(Pozzo Miller et al., 1995), or a calcium-activated non-specific cation (CAN) current (Crépel et al., 1994).

3.7.3 Inhibition of voltage-sensitive calcium channels (VSCCs)

A number of studies have demonstrated that application of mGluR agonists can inhibit voltage-sensitive Ca^{2+} channels (VSCCs) (see figure 3.5). This effect has been reported in many different cell types, including hippocampal, striatal, cortical and cerebellar granule cells. The pharmacological profile of this effect, and the susceptibility of N- and L-type calcium currents depends on the cell type or tissue studied (see Pin and Duvoisin, 1995; Conn and Pin, 1997 for review).

3.7.4 Modulation of ligand-gated ionotropic receptors

$GABA_A$, NMDA and AMPA receptors can all be regulated by mGluRs. (1*S*,3*R*)-ACPD inhibits $GABA_A$ receptor-mediated currents and potentiates AMPA-mediated responses in neurons of the rat tractus solitarus nucleus (Glaum and Miller, 1993). The profile of antagonism of these effects with phenylglycine derivatives suggests the involvement of group I mGluRs (Glaum et al., 1993).

In CA1 hippocampal slices, (1*S*,3*R*)-ACPD application has been reported to induce an NMDA receptor-independent potentiation of the AMPA receptor-mediated field EPSP (Bortolotto and Collingridge, 1993). (1*S*,3*R*)-ACPD has also been found to cause an acute enhancement of NMDA receptor-mediated currents in CA1 pyramidal cells, without affecting the AMPA-mediated component (Harvey et al., 1991; Anisztejn et al., 1992; Harvey and Colingridge, 1993). Anisztejn et al. (1992) reported that this upregulation of NMDA receptors was sensitive to PKC inhibitors, but negative results were obtained by Harvey and Collingridge (1993). This discrepancy may reflect the use of different PKC inhibitors by the two groups. The involvement of group I mGluRs is suggested by the finding that application of (*R*,*S*)-DHPG results in an acute enhancement of NMDA currents, whereas group II and III agonists are ineffective (Fitzjohn et al., 1996). The agonist-induced upregulation of NMDA receptor function in the above studies was limited to the period of ACPD application only. However, a long-term potentiation of NMDA receptor-mediated responses has been described in the dentate gyrus (O'Connor et al., 1994).

3.8 Presynaptic actions of mGluRs

3.8.1 mGluRs can act as presynaptic autoreceptors

A commonly observed effect of *trans*-ACPD application in hippocampal slice preparations was first reported by Baskys and Malenka (1991a). They found that a brief application of *trans*-ACPD decreased the initial slope of the CA1 field EPSP in a reversible and dose-dependent fashion. These findings have since been replicated by others (Pacelli and Kelso, 1991). Similar results had been obtained several years earlier using L-AP4 (Collingridge and Lester, 1989), a compound now known to be a selective mGluR group III agonist. Curiously, the presynaptic depression induced by the application of *trans*-ACPD is far more marked in neonatal rat brain slices than in adult slices (Baskys and Malenka, 1991b). In the same study, evidence was presented suggesting that the receptor mediating this depression in synaptic transmission is located pre-synaptically. Firstly, both the NMDA and AMPA receptor-mediated components of the EPSC were depressed by mGluR agonists. Secondly, responses to exogenous AMPA were unaffected, even when the EPSC was very strongly depressed. Thirdly, paired pulse facilitation, a pre-synaptic phenomenon, was enhanced during depression.

Manzoni and Bockaert (1995) have since shown that application of the group I selective agonist (*R*,*S*)-DHPG can induce a presynaptic depression of synaptic transmission at CA3-CA1 pyramidal cell synapses. The authors report that the application of the selective group II agonists L-CCG-I and 4-C3HPG had no effect on synaptic transmission. (1*S*,3*R*)-ACPD induced a similar presynaptic inhibition to DHPG, which was not blocked by the mGluR1 subtype preferring antagonist (*S*)-4CPG, suggesting that the effect is mediated by mGluR5. In a later study (Géreau and Conn, 1995a), the group II mGluR antagonist MCCG failed to inhibit the depressant actions of DHPG or L-AP4, confirming that group II mGluRs are not involved in this effect. In contrast, the mixed group I and II antagonist, (+)-MCPG blocked the depressant actions of DHPG, but was ineffective against the depression induced by L-AP4. Additional experiments confirmed that these agonist-induced depressant actions were indeed presynaptic in origin. These results suggest that the presynaptic autoreceptor-mediated actions of mGluR agonists in adult CA1 pyramidal cells are mediated by group I and III mGluRs, but not by group II.

Pharmacological studies of the neonatal hippocampal slice have revealed that, in contrast to the findings in adult slices, group II mGluRs can also act as presynaptic autoreceptors at an early developmental stage (Vignes et al., 1995). This result is consistent with the finding that the presynaptic depressant action of *trans*-ACPD is more pronounced in neonatal slices (Baskys and Malenka, 1991b).

It is curious that a group I mGluR, postulated to be mGluR5, should act as a presynaptic autoreceptor, since most immunolabelling studies have reported a postsynaptic location for this class of mGluR (e.g. Shigemoto et al., 1997). However, some immunoreactivity for mGluR5 has been reported at presynaptic terminals (Romano et al., 1995). Alternatively, an unidentified presynaptic mGluR5-like receptor may act as a group I autoreceptor (see section 3.8.2).

Group I and II agonists do not cause a reduction in synaptic transmission at lateral perforant path–dentate gyrus synapses. However, L-AP4 causes a marked depression which is blocked by the group III selective antagonist MAP4 (Bushell et al., 1995; Macek et al., 1996). The sensitivity of lateral perforant path synapses to micromolar concentrations of L-AP4, together with recent immunolabelling studies (Shigemoto et al., 1997), suggests that mGluR8 may act as the major autoreceptor in this pathway. Consistent with this possibility, (*R,S*)-MCPG has been reported to antagonize the presynaptic depression induced by L-AP4 in the lateral perforant path (Johansen et al., 1995), despite the fact that it is inactive at mGluRs 4 and 7 (Thomsen et al., 1994; Hayashi et al., 1994). However, a recent study suggests that MCPG acts as an antagonist of mGluR8 (Saugstad et al., 1996).

L-AP4 also depresses synaptic transmission at medial perforant path–dentate gyrus synapses, but with lower potency than in the lateral perforant path, consistent with the involvement of mGluR7 (Ugolini and Bordi, 1995; Macek et al., 1996). Group I selective agonists have no effect. However, application of selective group II agonists such as L-CCG-I and APDC causes a marked depression, which is blocked by antagonists of group II receptors. These results indicate that different receptor subtypes serve as autoreceptors at medial and lateral perforant path synapses.

3.8.2 A presynaptic mGluR increases glutamate release

In addition to acting as inhibitory autoreceptors, a presynaptic mGluR has been identified which increases glutamate exocytosis in cerebrocortical nerve terminals. Co-application of (1*S*,3*R*)-ACPD and arachidonic acid increased the 4-aminopyridine (4-AP)-induced release of glutamate form cerebrocortical synaptosomes (Herrero et al., 1992). This effect was sensitive to inhibition of PKC, suggesting that the increase in glutamate release is mediated by the synergistic activation of PKC by arachidonic acid and DAG generated by mGluR activation. Since arachidonic acid is a possible retrograde messenger (Lynch et al., 1991), mGluR-mediated increases in glutamate release may contribute to the expression of LTP. Indeed, Collins et al. (1995b) found that co-perfusion of (1*S*,3*R*)-ACPD and arachidonic acid in hippocampal slices evoked a rapid and long-lasting enhancement of synaptic transmission in CA1 hippocampal slices. The mGluR subtype which mediates this effect is unknown. It is possible that a currently unidentified presynaptic group I-like receptor is involved.

3.8.3 Summary

The studies reviewed in this section reveal that all groups of mGluRs can modulate presynaptic glutamate release. At CA3-CA1 synapses, group I and III mGluRs, but not group II, act as inhibitory presynaptic autoreceptors. However, group II and III receptors, but not group I, act as presynaptic autoreceptors in the medial perforant path–dentate gyrus projection. In addition to these inhibitory effects, there is evidence that the activation of a presynaptic group I-like mGluR in conjunction with arachidonic acid application results in an increase in glutamate release. The presynaptic actions of mGluRs are summarized in figure 3.5.

3.9 The role of mGluRs in synchronized network oscillations

In addition to their role in the oscillatory activity of individual neurons (e.g. McBain et al., 1994), there is evidence that mGluRs may be involved in the generation of synchronized neuronal network oscillations. Krieger et al. (1994) examined the effects of (1*S*,3*R*)-ACPD perfusion on both the single cell and network properties of the lamprey spinal cord. They found that (1*S*,3*R*)-ACPD induced a transient depolarization in all grey matter neurons tested, which could be reduced by the application of (+)-MCPG. The membrane depolarization was accompanied by an increase in synaptic noise attributed to an increase in the activity of presynaptic interneurons. Perfusion of the lamprey spinal cord preparation with NMDA results in burst firing of the neurons involved in the generation of locomotor activity, a phenomenon known as "fictive locomotion". Application of ACPD during perfusion with NMDA was found to increase the frequency of this locomotor rhythm, presumably due to an increase in the excitability of individual neurons within the network.

I.p.s.c. oscillations have also been seen in the hippocampus. Whittington et al. (1995) made intracellular recordings from CA1 pyramidal cells in a hippocampal slice preparation. In the presence of CNQX and D-AP5, single electrical stimuli evoked i.p.s.c.s oscillating at 40 Hz. Application of (+)-MCPG completely blocked these i.p.s.c. oscillations, but had no effect on monosynaptic i.p.s.c.s. Application of (1*S*,3*R*)-ACPD elicited 40 Hz i.p.s.c. oscillations similar to those evoked electrically; these oscillations were also blocked by MCPG. Similar ACPD-induced oscillations were seen in neocortical slices. The authors propose that tonic activation of mGluRs can provide the excitatory input to drive network oscillations of inhibitory interneurons, resulting in a 40 Hz oscillatory rhythm in pyramidal cells, which can entrain their firing.

The synchronous firing of hippocampal or cortical cells has been proposed as a solution to the problem of how information carried by different populations of cells, located at some distance from each other, can be associated to form a coherent percept, the so-called "binding problem". Whittington et al. (1995) propose that the long-range coupling necessary to achieve this oscillatory coherence could occur via intercortical interconnections or via the thalamocortical loop.

A slightly different approach was taken by Boddeke et al. (1997), who recorded spontaneous field potential activity close to the CA1 pyramidal cell layer. Application of (1*S*,3*R*)-ACPD or quisqualate caused a large increase in spontaneous activity. Analysis of power spectra revealed a mean dominant frequency of roughly 20 Hz. This rhythm was distinct from the 9 Hz "theta-like" rhythm induced by application of the muscarinic agonist carbachol. The firing of individual CA1 pyramidal cells in response to ACPD was phase-locked to the 20 Hz cycle, but the firing rates of single cells increased to only 8.2 Hz after drug infusion. However, multiple unit firing rates increased to frequencies between 15 and 25 Hz, indicating that the dominant 20 Hz rhythm is an emergent network property,

rather than a simple reflection of unit activity. Synchronized activity persisted in the presence of ionotropic glutamate receptor antagonists. However, application of the $GABA_A$ antagonist bicuculline strongly reduced the rhythmic activity induced by ACPD, suggesting a prominent role of GABAergic interneurons in the generation of these oscillations. In contrast, the cholinergic theta rhythm is enhanced after administration of bicuculline (Konopacki et al., 1987). The authors suggest that the 20 Hz oscillations described are generated by a similar $GABA_A$ receptor-dependent mechanism to that described by Whittington et al. (1995). mGluR agonist-induced rhythmic activity was inhibited by (+)-MCPG. The potency rank order of the two agonists was quisqualate > (1*S*,3*R*)-ACPD. Since quisqualate is a more potent agonist at group I mGluRs than (1*S*,3*R*)-ACPD, but a considerably weaker agonist than (1*S*,3*R*)-ACPD at group II mGluRs, it is likely that synchronous the 20 Hz activity is induced by the activation of group I mGluRs.

Another form of oscillatory activity was described by Taylor et al. (1995) in rat hippocampal CA3 pyramidal cells. Application of a high concentration of (1*S*,3*R*)-ACPD induced trains of depolarizations with superimposed action potentials. Individual trains lasted for several seconds, and depolarization frequencies slowed from an initial maximum of 27 Hz to a frequency of 8 Hz towards the end of the train. This activity was found to be synchronous throughout the CA3 pyramidal cell population. Oscillations were blocked by the application of the mGluR antagonists (+)-MCPG, (*S*)-4C3HPG and (*S*)-4CPG. Since the latter two drugs are group I mGluR antagonists, but group II agonists, it is likely that PI-coupled mGluRs are responsible for the oscillatory activity. However, unlike the rhythmic activity described previously, these oscillations persisted in the presence of $GABA_A$ antagonists, but were blocked by the application of CNQX. The authors speculate that this form of rhythmic activity could be mediated by recurrent excitation, and may be more relevant to the pathophysiology of epileptiform discharges, than the generation of physiological rhythmic activity.

3.10 The role of mGluRs in hippocampal long-term potentiation

3.10.1 NMDA receptor activation alone is insufficient to induce LTP

It is well established that NMDA receptor activation during a tetanus is necessary for the induction of LTP in the dentate gyrus and area CA1 of the rodent hippocampus, since potentiation is completely blocked, both *in vivo* and *in vitro*, by the application of the selective NMDA receptor antagonist, AP5 (Collingridge et al., 1983; Harris et al., 1984; Morris et al., 1986a; Errington et al., 1987). However, direct iontophoresis of NMDA in hippocampal slice preparations induces only a short-lasting potentiation which decays back to baseline values within about 30 min (Collingridge et al., 1983; Kauer et al., 1988a). In contrast, potentiation induced by a high frequency tetanus consists of a short-lasting decremental phase similar to that induced by NMDA, followed by a stable increase in synaptic efficacy which may remain above baseline values for hours or even days (see chapter 2.4.2).

Early studies demonstrated that perfusion of a conditioning solution containing glutamate, low Mg^{2+} and high K^{+} could induce a non-tetanic form of LTP in guinea pig CA1 slices (Izumi and Kato, 1987a). If glutamate was replaced by NMDA, LTP was not observed, suggesting that activation of NMDA receptors alone is not sufficient for the induction of LTP. However, if quisqualate was administered prior to NMDA application, LTP was again induced (Izumi et al., 1987a). It has been shown that tetanization of an afferent pathway leads to an elevation of intracellular calcium in hippocampal slices which lasts for several minutes (Kudo et al., 1987). Perfusion of a low Ca^{2+} medium starting immediately after tetanization and lasting for 5 min blocks LTP (Izumi et al., 1987b). However, other studies have found that the voltage dependent Ca^{2+} influx (via NMDA receptors and VSCCs) is not sufficient to account for the duration of the elevation of intracellular calcium following a tetanus (Kudo et al., 1987; Krnjevic et al., 1986). The necessity for an additional Ca^{2+} source was further suggested by the finding that tetanization did not result in LTP in the presence of an inhibitor of Ca^{2+} release from intracellular stores (Izumi et al., 1990). On the basis of these results, it was proposed that activation of the newly identified quisqualate metabotropic receptor, known to stimulate IP3 synthesis and the release of intracellular Ca^{2+}, might provide the extra trigger necessary for the induction of stable LTP (Izumi et al., 1990).

3.10.2 The effects of AP3 and AP4 on LTP induction

Early studies were carried out with the putative antagonist of PI turnover, AP4 (see section 3.6.2). Reymann and Matthies (1989) reported that, in hippocampal CA1 slices, a late phase of LTP is reportedly blocked by the application of this drug. However, D-AP4, which does not inhibit PI

turnover, was found to be as effective as L-AP4, the active isomer, a result which raised serious doubts as to the selectivity of the drug. Furthermore, D-AP4 applied shortly after the tetanus was almost as effective as when applied before tetanization. L-AP4 is now known to be a selective agonist of group III mGluRs, although the mechanism by which it inhibits PI hydrolysis is unknown.

The weak partial mGluR antagonist AP3 has also been used in a number of studies. Izumi et al. (1991) found that D,L-AP3 blocked both PTP and LTP in CA1 slices. The application of AP3 immediately after tetanization was also found to limit the duration of LTP. Behnisch et al., (1991) found that D,L-AP3 could block late LTP, and was effective even when applied after the tetanus. However, the D-isomer of AP3 is a weak NMDA receptor antagonist, so these results must be viewed with caution. Nevertheless, a subsequent study revealed that a dose of L-AP3 demonstrated to have no effect on ionotropic receptor-mediated EPSCs could nevertheless block the late phase of EPSP slope potentiation (Behnisch and Reymann, 1993). Since similar results had been obtained with the application of PKC inhibitors (Reymann et al., 1988a, b), it was postulated that the action of AP3 was mediated by inhibition of PKC resulting from the blockade of PLC-coupled mGluRs.

Similar results were presented by Zheng and Gallagher (1992), who found that moderate doses of AP4 and AP3 produced a stereoselective block of LTP in the dorsolateral septal nucleus, a region that has the highest density of mGluR-like binding sites in the rat brain (Cha et al., 1990), and in which LTP induction is not affected by the application of D-AP5. In contrast, Stanton et al. (1991) reported that L-AP3 blocked the induction of homosynaptic LTD induced by the out of phase pairing of test and conditioning inputs to CA1 pyramidal cells (see chapter 2.5.1). The induction of homosynaptic LTP by an in phase pairing protocol was not blocked. However, LTP was assessed 30 min after induction, and the late phase of LTP found to be blocked by Behnisch and Reymann (1993) was not assessed.

3.10.3 The effect of MCPG on LTP induction

Despite its inhibition of PI turnover, L-AP3 does not affect certain electrophysiologically determined mGluR-mediated responses in the hippocampus (Stratton et al., 1990; Charpak and Gäwiler, 1991; Hu and Storm, 1992; Desai et al., 1992). This problem was resolved following the characterisation of a weak, but selective and competitive antagonist, (*R,S*)-α-methyl-4-carboxyphenylglycine (MCPG) (see sections 3.6.2 and 3.6.3).

MCPG has been widely used since its discovery, but studies of its effects on the induction of LTP have often yielded contradictory results. The first report of the ability of MCPG to block hippocampal LTP was published soon after the initial characterization of the drug. It was found that 500 μM (*R,S*)-MCPG was sufficient to block LTP in CA1 hippocampal slices, sparing only a short-

term potentiation lasting about 30 min (Bashir et al., 1993). In this study, application of L-AP3 produced no such block, even after 3 hours. The efficacy of (*R,S*)-MCPG as an mGluR antagonist was demonstrated when calcium-mobilizing responses to (1*S*,3*R*)-ACPD in isolated mGluR1a-transfected cells devoid of ionotropic glutamate receptors were found to be reversibly abolished. MCPG was also shown to reverse the ACPD-induced inhibition of action potential accommodation and after-hyperpolarization in hippocampal CA1 pyramidal cells. A similar result was reported by Sergueeva et al. (1993) who found that 500 μM (*R,S*)-MCPG limited the duration of LTP to little more than an hour, as well as preventing the tetanus-induced increase in AMPA receptor sensitivity. (*R,S*)-MCPG has been found to block both NMDA receptor-dependent and NMDA-receptor independent LTP in CA1 slices (Little et al., 1995). The latter form of LTP is dependent on VSCC activation. Recently, Breakwell et al. (1996) reported that (+)-MCPG could block EPSP slope, population spike, and E-S LTP in rat hippocampal slices. A large STP remained, which decayed to baseline within 30 min.

MCPG has been found to limit LTP induction *in vivo*. Riedel and Reymann (1993) reported that an i.c.v. injection of MCPG could limit the duration of LTP to 2-3 hr in freely moving rats. In a later paper, the same authors reported a complete block of both STP and LTP by a tenfold higher concentration of MCPG (Riedel et al., 1994a). The (-)-form of MCPG was found to be ineffective. Richter-Levin et al. (1994) reported a reduction in EPSP slope potentiation 1 hr after tetanization following bilateral i.c.v. infusions of (*R,S*)-MCPG under urethane anaesthesia. However, continuous perfusion of (*R,S*)-MCPG via a push-pull cannula for 30 min prior to tetanization resulted a complete block of both EPSP slope and population spike LTP 2 hr after induction.

Despite these positive reports, Chinestra et al. (1993) reported a failure of (*R,S*)-MCPG to block LTP in CA1 hippocampal slices. However, the authors also found that MCPG failed to antagonize the block of slow after-hyperpolarization and action potential accommodation induced by (1*S*,3*R*)-ACPD (see Bashir et al., 1993). Furthermore, no blockade of the ACPD-induced slow inward current thought to result from the inhibition of potassium conductances, or the depression in field potentials induced by a higher concentration of (1*S*,3*R*)-ACPD was observed. These latter results have led to considerable scepticism concerning the reported absence of an effect on LTP. However, others have reported that MCPG has no inhibitory effect on LTP in areas CA1 and CA3 of the guinea-pig hippocampus (Manzoni et al., 1994). Nevertheless, contrary to the findings of Ben-Ari's group, MCPG was able to antagonize the blocking action of ACPD on slow after-hyperpolarization in CA1, as well as reducing the presynaptic inhibitory actions of ACPD on field EPSPs in both CA1 and CA3. Other workers have found that MCPG does not block LTP in the dentate gyrus of urethane-anaesthetized rats (Bordi and Ugolini, 1995).

3.10.4 The "molecular switch" hypothesis

The reasons for the contradictory findings on the ability of MCPG to block LTP are not yet understood, although it is likely that differences in experimental procedure may account for some of the discrepancies. One potential explanation for the mixed results is provided by the findings of Bortolotto et al. (1994) who reported that in CA1 slices, (*R*,*S*)-MCPG failed to block LTP in pathways which had previously undergone sub-maximal potentiation. The same result was obtained if the first "conditioning" tetanus was delivered in the presence of D-AP5 which completely blocks LTP, or if the conditioning tetanus was replaced by the application of (1*S*,3*R*)-ACPD. These data suggest that the activation of mGluRs turns on a "molecular switch" which eliminates the requirement for further mGluR activation in the induction of LTP. Activation of the switch could be prevented by the application of MCPG or a protein kinase inhibitor, K-252b during the conditioning train. The conditioning effect was found to last up to 6 hr. However, low-frequency stimulation which induced depotentiation following the conditioning tetanus was found to re-establish the ability of MCPG to block LTP after a second tetanus. This "deconditioning" effect was also mGluR-mediated, since application of MCPG during low frequency stimulation abolished the re-setting of the switch.

It has been suggested that certain techniques for cutting hippocampal slices may effectively tetanize the slice prior to the start of an experiment, thus rendering mGluR activation unnecessary for subsequent LTP. Consistent with this possibility, it was reported that in dentate slices, (*R*,*S*)-MCPG was only able to block LTP of the AMPA-mediated EPSC in response to tetanization of the associational / commissural pathway after the delivery of low frequency stimulation resulting in LTD (Wang et al., 1995). Negative results have, however, been reported by Selig et al. (1995) and Thomas and O'Dell (1995), who failed to see a block of LTP in CA1 slices in the presence of (*R*,*S*)-MCPG, even after low frequency stimulation of afferents, although in the latter study, MCPG was found to inhibit the LTP induced with a weak induction protocol.

3.10.5 The role of tetanus parameters in determining the sensitivity of LTP to MCPG

It seems that the molecular switch hypothesis cannot account for all failures of MCPG to block LTP. However, Thomas and O'Dell's result suggests that tetanization strength may be critical. This possibility is consistent with the finding that the LTP induced in area CA1 by certain tetanization protocols such as theta burst stimulation is not blocked by MCPG (Brown et al., 1994). Furthermore, it has recently been reported that MCPG only results in a block of LTP induced by weak tetanization, owing to the requirement for calcium release from intracellular stores under these circumstances. After strong tetanization, this requirement is bypassed by calcium influx via voltage-dependent calcium channels (Wilsch et al., 1998).

The issue of tetanization strength may also explain why MCPG fails to block LTP under urethane anaesthesia (Bordi and Ugolini, 1995; Martin and Morris, 1997), but not in freely moving rats (Riedel et al., 1995a). For instance, it has been found that stronger tetanization parameters are required to induce LTP in area CA1 of urethane-anaesthetized rats, compared to the freely moving state (Riedel et al., 1994b). Similarly, *Thy-1* knockout mice show a complete block of dentate LTP under urethane anaesthesia, but express significant LTP in the freely moving state (Nosten-Bertrand et al., 1996; Errington et al., 1997). The weaker tetanization parameters necessary to induce LTP in freely moving animals may unmask a modulatory role of mGluRs. Nevertheless, studies involving MCPG do not support an obligatory role for mGluRs in LTP induction, despite the fact that there may be circumstances in which a modulatory function is revealed. However, there are a number of problems with the use of MCPG as an mGluR antagonist; some these were discussed in section 3.6.3, and others will be addressed in the following two sections.

3.10.6 mGluR subtype-specific antagonists and the inhibition of LTP

MCPG is not a subtype-selective antagonist, and several newer compounds with subtype-specific actions have now been tested. Nevertheless, such studies have mostly generated negative results. For instance, Bordi and Ugolini (1995) found that, in addition to MCPG, the preferential mGluR1 antagonist (*S*)-4CPG had no effect on the induction of LTP *in vivo*. A similar result has been obtained with the recently synthesized group I-selective agonist AIDA (Hölscher et al., 1997b; McCaffery et al., 1998). The group II antagonists MSOPPE (Manahan-Vaughan, 1997) and MCCG (Hölscher et al., 1997b) also fail to block LTP *in vivo*, as does the group III antagonist MAP4 *in vitro* (Breakwell et al., 1998). In the same study, it was found that CA1 LTP could be induced in the presence of a cocktail of (*S*)-4CPG, MCCG and MAP4, antagonists at groups I, II and III respectively. No biochemical assays for the antagonism of mGluR-mediated second messenger systems were carried out in the above studies. Nevertheless, the data suggest that mGluR antagonists do not prevent the induction of LTP. However, currently available antagonists are rarely selective for all members of a group. Hence, although it is likely that the activation of mGluR1 is not obligatory for the induction of LTP, the role of mGluR5 is currently uncertain, since MCPG and 4CPG are inactive at mGluR5 (Joly et al., 1995), and AIDA has been found to a much weaker antagonist at mGluR5a compared to mGluR1a (Moroni et al., 1997).

3.10.7 Group II and III mGluR agonists and the inhibition of LTP

It has been reported that the selective group III mGluR agonist L-AP4 blocks LTP in the dentate gyrus and CA1 of 12-week-old rats; by this age, L-AP4 has no effect on baseline synaptic

transmission in either region (Manahan-Vaughan and Reymann, 1995c). The authors suggest that L-AP4 may exert its effects by inhibiting glutamate release during tetanization. These results are consistent with earlier reports that L-AP4 could limit the duration of LTP, originally attributed to the inhibition of PI turnover.

Curiously, the group II mGluR agonist (1*S*,3*S*)-ACPD has also been reported to block both dentate and CA1 LTP *in vivo* (Hölscher et al., 1997a, b). However, (1*S*,3*S*)-ACPD also acts as a less potent agonist at group I and III mGluRs. Nevertheless, a block of LTP has been reported even with low concentrations of (1*S*,3*S*)-ACPD, an effect which was prevented by co-perfusion with MCCG, suggesting that an agonist action at group II mGluRs may indeed be responsible for the block of LTP (Hölscher et al., 1997b). Surprisingly, the block of LTP with (+)-MCPG was also prevented by the group II mGluR antagonist MCCG in CA1 slices (Breakwell et al., 1998): since antagonists of mGluR groups I, II and III were completely ineffective in blocking LTP, it was proposed that MCPG blocks LTP via an agonist action at a group II, or group II-like, mGluR.

Independent studies have suggested that MCPG may have agonist properties at one or more mGluR subtypes. It was found that MCPG activated a presynaptic mGluR autoreceptor in the basolateral amygdala, although pharmacological characterization of the subtype involved was not carried out (Keele et al., 1995). Application of MCPG in rat dentate slices has been reported to induce a depression in field EPSPs, an effect which was attributed to an agonist action at group II mGluRs (Huang et al., 1997). However, several lines of evidence suggest that MCPG does not have agonist actions at any known group II mGluR subtype. For example, MCPG antagonizes the presynaptic depression induced by (1*S*,3*S*)-ACPD in the dentate gyrus and neonatal CA1, an effect thought to be mediated by the activation of presynaptic group II mGluRs. Moreover, mGluRs 2 and 3 are largely absent in adult area CA1, suggesting that the block of LTP by putative group II agonists in this region is unlikely to be mediated by a known subtype (see section 3.4.2). In addition, MCPG has not been found to be an agonist of cloned group II mGluR subtypes studies in expression systems (Hayashi et al., 1994; Emile et al., 1996). However, (*S*)-4CPG, which does act as an agonist at mGluR2, in addition to antagonizing mGluR1, has no effect on LTP (Bordi and Ugolini, 1995). The pharmacological characterization of mGluR3 is currently poor, but this receptor is absent in CA1 and only weakly expressed in the dentate, making it an unlikely modulator of LTP (see section 3.4.2). Hence, it is possible that MCPG blocks LTP via an action at an unidentified mGluR subtype with group II-like pharmacology. It is likely that the cloning of additional mGluR subtypes together with the arrival of new pharmacological tools will help to resolve this issue in the near future.

3.10.8 mGluR activation can enhance tetanically induced LTP

Early studies revealed that the magnitude of tetanus-induced LTP in area CA1 *in vitro* could be greatly increased by prior application of *trans*-ACPD (McGuinness et al., 1991a, b; Otani and Ben-Ari, 1991). It was also reported that a weak tetanus, normally resulting only in STP, induced LTP when delivered after *trans*-ACPD application (Aniksztein et al., 1991; Behnisch and Reymann, 1993). In the former study, application of ACPD was found to potentiate NMDA, but not AMPA-mediated EPSCs, suggesting a possible mechanism for the enhancement of tetanic LTP: this up-regulation of NMDA receptor was blocked by the application of a PKC inhibitor. In a later study, coupling of a weak tetanus with ACPD application was found to result in a transient elevation of cytosolic PKC activity, consistent with previous suggestions that PKC activation may be involved in the induction of lasting LTP (Otani et al., 1993).

A similar facilitation of LTP induction has been seen with the putative group I-selective mGluR agonist *t*-ADA in the dentate gyrus of freely-moving rats (Riedel et al., 1995c). However, negative results were obtained in the dentate gyrus of rat hippocampal slices with both *t*-ADA, and DHPG (Brown and Reymann, 1995). The reasons for this discrepancy are unknown, but differences between *in vivo* and *in vitro* preparations may be relevant. A later study from the same laboratory revealed that the activation of mGluRs with *t*-ADA could overcome an AP5-induced block of LTP in the dentate gyrus of freely-moving rats, in addition to facilitating the induction of lasting LTP with a weak tetanus in the absence of AP5 (Manahan-Vaughan et al. 1996). Furthermore, it was found that application of *t*-ADA or DHPG could still facilitate the induction of LTP when applied up to 25 min after tetanization (Manahan-Vaughan and Reymann, 1996). In view of these findings, it is unlikely that an mGluR-mediated upregulation of NMDA receptors underlies this form of LTP enhancement. However, the discrepancy between these results and those of other studies remains unresolved. Nevertheless, the ability of mGluR activation within a limited time window after tetanization to extend the duration of LTP is reminiscent of similar findings following the activation of dopaminergic or β-adrenergic receptors, a phenomenon which is consistent with the "synaptic tagging" theory of late LTP induction (Frey and Morris, 1998).

The proposed role of group I mGluRs in the facilitation of LTP induction is difficult to reconcile with the failure of group I-selective antagonists to block LTP. It is possible that the activation of mGluR5, for which few mGluR antagonists have significant affinity, may explain the discrepancy: DHPG has been reported to be an effective mGluR5 agonist (Brabet et al., 1995; Géreau and Conn, 1995b), and *t*-ADA has 10-fold greater potency at mGluR5 than mGluR1 (Manahan-Vaughan et al., 1996). Alternatively, the mGluR-mediated blockade and facilitation of LTP may be mechanistically distinct, perhaps involving different subtype populations. It is likely that new mGluR ligands selective for individual subtypes will provide answers to these questions.

3.10.9 Summary

These results suggest that mGluRs play a non-obligatory modulatory role in the induction of LTP, but that the nature and mechanism of this role may vary between brain regions and between experimental preparations. Methodological issues such as tetanus strength and anaesthesia have been suggested to explain discrepancies between studies. In addition, the molecular switch hypothesis describes circumstances in which mGluR activation is not involved in the induction of LTP. These issues will be revisited in chapter 7, in which a series of experiments concerning the effect of MCPG on dentate LTP *in vivo* are described.

3.11 mGluR agonists and "slow-onset potentiation"

3.11.1 Slow-onset potentiation in vitro

Several groups have reported that co-activation of mGluRs and NMDA receptors induces a lasting potentiation. For instance, Izumi et al. (1987b) found that co-application of quisqualate and NMDA in CA1 slices caused a lasting LTP, not induced by NMDA application alone, a result which was later interpreted as suggesting a role for quisqualate-activated mGluRs in LTP (Izumi et al. 1990). Radpour and Thomson (1992) subsequently found that co-application of trans-ACPD and NMDA could induce a slowly developing potentiation in CA1 slices, but only when accompanied by low frequency stimulation of afferents. This effect was not dependent on postsynaptic depolarization, or a block of synaptic inhibition.

However, other groups have found that activation of mGluRs may in itself be sufficient to induce a form of long-term potentiation, equivalent to the maintenance phase of tetanic LTP without the prior occurrence of STP or PTP. Bortolotto and Collingridge (1992, 1993) reported that the application of (1*S*,3*R*)-ACPD could induce a slow-onset potentiation in area CA1 which totally occluded tetanus-induced LTP, but was not itself dependent on NMDA receptor activation. The dose of ACPD used to produce this effect had no effect on the baseline EPSP, suggesting that presynaptic depression of glutamate release, and postsynaptic depolarization resulting from the inhibition of K^+ currents were minimal. (1*S*,3*R*)-ACPD-induced LTP was prevented by the application of protein kinase inhibitors or the intracellular Ca^{2+} ATPase inhibitor thapsigargin, suggesting that both arms of the phosphoinositide second messenger system may be involved. Interestingly, removal of CA3 prevented ACPD-induced slow-onset potentiation, suggesting that some form of ongoing synaptic activity may be necessary.

The necessity for intact connections to CA3 may explain the negative results obtained by other groups, since CA3 is often removed during preparation of a CA1 slice. For instance, Collins and Davies (1993) found that co-application of NMDA and (1*S*,3*R*)-ACPD was necessary to induce slow-onset potentiation in CA1 slices from which CA3 had been removed. A subsequent study revealed that ACPD alone was sufficient if intact CA3-CA1 connections were preserved (Collins et al., 1995a). However, it was found that infusion of ACPD was associated with an increase in the excitability of CA3 neurons, the appearance of spontaneous burst firing in CA3, and an increase in the presynaptic fibre volley. A similar form of "slow-onset potentiation" had previously been reported by Chinestra et al. (1994), and attributed to an increase in the presynaptic fibre volley. Collins et al. (1995a) found that the effects of ACPD on CA3 excitability and the afferent fibre volley to CA1 – together with slow-onset potentiation itself – were blocked by the application of D-AP5, in contrast to the findings of Bortolotto and Collingridge (1993). These results suggest that

ACPD-induced slow-onset potentiation is not mediated by an increase in synaptic efficacy such as that induced by a high frequency tetanus, but may be an indirect effect of an increased presynaptic fibre volley from CA3. However, it is also possible that burst firing originating from CA3 might constitute a physiological tetanus resulting in NMDA receptor-dependent LTP at synapses in CA1 (see Collins et al., 1995a).

Additional evidence against a postsynaptic mechanism for the induction of slow-onset potentiation was provided by Liu et al. (1993) who found that application of (1*S*,3*R*)-ACPD could induce an LTP of AMPA-receptor mediated EPSPs which was secondary to an LTD of GABAergic IPSPs.

These issues were addressed in a series of experiments by Bortolotto and colleagues, who provided a detailed characterization of the effect they had originally described (Bortolotto and Collingridge, 1995). Firstly, the addition of picrotoxin did not prevent slow-onset potentiation, suggesting that the effect is not secondary to a depression of synaptic inhibition. Secondly, ACPD-induced potentiation was not associated with an increase in the presynaptic fibre volley. In addition, the expression of slow-onset potentiation was found to be mediated entirely by an increase in AMPA receptor mediated EPSPs. In contrast to the findings of O'Connor et al. (1994), no potentiation of the pharmacologically isolated NMDA receptor EPSP was observed. It was found that the delivery of a high-frequency tetanus in the presence of AP5 could substitute for the presence of CA3, suggesting that high frequency burst activation is necessary for the induction of ACPD-mediated slow-onset potentiation, although the mechanism involved is NMDA receptor-independent. These findings demonstrate that the slow-onset potentiation observed by Bortolotto and colleagues is mechanistically distinct from that described by Chinestra et al. (1994), and Collins and Davies (1994). The former class of slow-onset potentiation is clearly more similar to the maintenance phase of tetanic LTP than the latter, but the extent to which the two forms of potentiation are mechanistically equivalent remains unknown.

Methodological considerations may explain some of the discrepancies between the effects of (1*S*,3*R*)-ACPD in different laboratories. One such possibility concerns the use of interface slice chambers by some groups, and submersion chambers by others. For instance, Bortolotto et al. (1995) found that switching from an interface to a submerged slice configuration prevented the occurrence of slow-onset potentiation. A slight acidification of the tissue found to occur after submersion of the slice was suggested as a possible explanation. However, this does not explain why other groups have obtained a mechanistically different form of slow-onset potentiation using submersion slice chambers (e.g. Chinestra et al., 1994). In fact, the use of an interface chamber has been criticised by Chinestra et al. (1994) for being "less than physiological", since slices are exposed to oxygenated air rather than perfused with aCSF.

Although these issues remain largely unresolved, recent experiments have provided evidence for a form of mGluR-induced potentiation which, though not identical to that reported by Bortolotto and

colleagues, appears to be postsynaptic in origin and may be mechanistically related to the maintenance phase of tetanically-induced LTP. For instance, (1*S*,3*R*)-ACPD was found to induce a transient depression, followed by a fairly rapid potentiation of both AMPA and NMDA receptor-mediated EPSCs in rat dentate granule cells *in vitro* (O'Connor et al., 1995). This effect was found to be AP5-sensitive. A similar result was reported by Breakwell et al. (1996), who found that bath application of (1*S*,3*R*)-ACPD induced a transient depression, followed by a fairly rapid EPSP slope and E-S potentiation in submerged rat CA1 slices. Potentiation was prevented by the application of AP5, and was only observed when an intact CA3 connection was present. However, no increase in the presynaptic fibre volley was seen following ACPD application. This suggests that a change in the excitability of CA3 neurons does not underlie the present result, although the exact role of CA3 is unknown. E-S potentiation was blocked by the application of picrotoxin, whilst synaptic potentiation was spared, suggesting that ACPD induced E-S potentiation, but not EPSP slope potentiation, may result from a depression of GABAergic inhibitory transmission such as that reported by Liu et al. (1993). Application of (+)-MCPG blocked the EPSP slope and E-S potentiation induced by both ACPD application and high frequency tetanization. A similar, but NMDA receptor-independent, form of potentiation has recently been described following the application of the selective group I mGluR agonist (*S*)-DHPG in dentate slices (O'Leary and O'Connor, 1997). This potentiation occluded tetanically-induced LTP, and *vice versa*. No change in the presynaptic fibre volley was seen after (*S*)-DHPG application.

It is possible that the potentiation observed in the above studies, particularly the final one, may be mechanistically similar to that reported by Bortolotto et al. (1995). The initial depression and faster time course of potentiation in these later studies may result from the use of slices from immature rats. The choice of hippocampal region, i.e. CA1 or dentate, is also likely to be relevant. However, there is still no consensus regarding the induction of LTP by the activation of mGluRs. For instance, a recent study found that application of (1*S*,3*R*)-ACPD in immature rat CA1 slices resulted in LTD, whereas effects in adult rats were variable, ranging from minimal change to slow-onset potentiation (Overstreet et al., 1997). In fact, there are currently almost as many forms of slow-onset potentiation described in the literature as there are laboratories investigating the phenomenon.

3.11.2 Slow-onset potentiation in vivo

Slow-onset potentiation following the infusion of mGluR agonists has sometimes been described *in vivo*. Manahan-Vaughan and Reymann (1995a) found that i.c.v. infusion of (1*S*,3*R*)-ACPD induced a slow onset potentiation in the dentate gyrus of freely-moving rats. This effect was completely blocked by the application of D-AP5 or (*R*,*S*)-MCPG. A similar result was obtained in area CA1 of freely moving rats using either (1*S*,3*R*)-ACPD (Manahan-Vaughan and Reymann, 1995b), or the putative group I mGluR agonists DHPG and *t*-ADA (Manahan-Vaughan and Reymann, 1997a). No

alterations in EEG such as epileptiform activity, or behavioural changes such as "wet dog shakes" were found after ACPD infusion in the former study. An independent report confirmed that (1*S*,3*R*)-ACPD can induce a slow onset potentiation in area CA1 of urethane-anaesthetized rats, which was found to occlude tetanus-induced LTP (Davis and Laroche, 1996). No changes in the afferent fibre volley were induced by ACPD. In the same study, however, a dose-dependent biphasic effect was obtained in the dentate gyrus, consisting of a short-term potentiation induced by both low and high doses of ACPD, followed by a lasting depression of the response in the high-dose group only. Since (1*S*,3*R*)-ACPD is a broad spectrum agonist acting at both group I and group II mGluR sub-types, it is possible that the potentiation of the EPSP is mediated by class I mGluRs coupled to PI hydrolysis, whereas the depression is mediated by presynaptically located class II mGluRs. However, this interpretation is difficult to reconcile with the fact that (1*S*,3*R*)-ACPD has a higher affinity for group II mGluRs than group I. Nevertheless, the differential effects of ACPD in the dentate versus CA1 might reflect the fact that group II receptors are not prominently expressed in CA1, and do not play a role as autoreceptors in this region (see sections 3.4.2 and 3.8.1, respectively). Consistent with this possibility, the group I mGluR-selective agonist (*R*,*S*)-DHPG induced only a potentiation of synaptic transmission in the dentate, whereas the mixed group II agonist / group I antagonist (*S*)-4CPG caused only a depression. Interestingly, application of a high dose of (1*S*,3*R*)-ACPD and (*S*)-4CPG blocked the subsequent induction of tetanic LTP, providing further evidence that activation of a receptor or receptors with group II-like pharmacology can block LTP.

Despite the fact that the *in vivo* data described above provide some support for the *in vitro* reports of slow-onset potentiation, such data must be interpreted with caution. Although no evidence of a presynaptic excitability or epileptiform activity was reported in these studies, other confounding factors not monitored, such as brain temperature, may contribute to an apparent slow-onset potentiation (c.f. Erickson et al., 1993). In fact, a number of glutamate receptor agonists, including L-glutamate, NMDA and (1*S*,3*R*)-ACPD have been reported to cause a dose-dependent dilation of hippocampal microvessels (Fergus and Lee, 1997). The increase in local blood flow resulting from this phenomenon would very likely lead to a temperature rise, an effect which may contribute to reports of slow onset potentiation induced by ACPD.

In summary, there is considerable evidence that activation of mGluRs, sometimes in conjunction with NMDA receptors, can cause a potentiation of hippocampal evoked responses via a pre- or postsynaptic mechanism, depending on the preparation used. However, it remains unknown whether these effects have any physiological relevance in the intact animal.

3.12 The role of mGluRs in learning

A number of groups have reported that the manipulation of mGluR function by pharmacological or genetic means can cause learning deficits, a result which is not surprising considering the wide regional distribution and diverse physiological roles of mGluRs. A satisfactory account of mGluR function would require a detailed understanding of the role of the individual mGluR subtypes within a particular brain structure during the performance of a specific behavioural task. For example, it has been demonstrated that the activation of mGluR2 in the accessory olfactory system in mice can lead to the induction of an olfactory memory (Kaba et al., 1994). However, such unambiguous results are rare, particularly when more complex forms of learning are studied.

3.12.1 Effect of mGluRs in memory consolidation in chicks and rats

Initial work revealed that injection of MCPG into the chick IMHV region prior to the acquisition of an inhibitory avoidance task could prevent long-term memory consolidation (1-2 hr) without affecting retention at shorter intervals. The onset of amnesia was dose dependent, with higher concentrations of drug resulting in faster forgetting (Hölscher, 1994; Rickard and Ng, 1995). MCPG has also been found to impair the long-term consolidation of a visual discrimination task in young chicks, a task in which NMDA receptor antagonists have no effect (Tiunova et al., 1996). In addition, Bianchin et al. (1994) found that bilateral intrahippocampal infusion of MCPG immediately after training produced a retention deficit in a step-down inhibitory avoidance task in rats. No amnesia was seen when MCPG was administered 180 min after acquisition, indicating that mGluR activation immediately after, or during training is critical for memory formation. Application of (1*S*,3*R*)-ACPD together with MCPG resulted in normal retention of the task, whereas ACPD alone caused an enhancement of memory. These results suggest that the level of mGluR activation immediately after learning can modulate the strength of the ensuing memory trace.

3.12.2 mGluRs and fear conditioning in rats

Aiba et al. (1994) reported that mice lacking mGluR1 were moderately impaired in contextual fear conditioning. However, intraventricular infusion of MCPG has subsequently been found to have no effect in this task, in comparison with the NMDA receptor antagonist D-AP5 which caused a significant deficit (Bordi et al., 1996). Nevertheless, application of the selective group I mGluR antagonist AIDA has recently been found to cause a selective deficit in contextual conditioning, while hippocampal independent cue conditioning was unaffected (Nielsen et al., 1997). Lu et al.,

(1997) have since reported that mice lacking mGluR5 exhibit a contextual conditioning impairment. Further evidence for a role of mGluR5 in contextual fear conditioning has recently been provided by Casabona et al., (1997a) who found that mGluR5 protein levels were markedly increased in area CA3 24 hr after contextual fear conditioning. 10 days later, CA3 expression had diminished, but protein levels were elevated in area CA1. Little change in mGluR5 protein was observed in the dentate gyrus, however.

Intraperitoneal injection of the group II selective mGluR agonist APDC prior to the acquisition of fear conditioning caused a severe deficit in freezing to a context, but not to a simple cue. This deficit could be eliminated by increasing the salience of the context by placing black and white stripes on the walls of the conditioning chamber and introducing the odour of eucalyptus oil. Increasing the salience of the US by raising the shock intensity also abolished the APDC-induced deficit (Riedel et al., 1997). These data are interpreted as evidence that excessive mGluR activation constitutes "noise" which prevents efficient learning. It is proposed that an increase in the "signal", in this case the CS or US, can overcome the increase in noise, thus permitting learning to occur normally. However, it could also be argued that activation of presynaptic group II mGluRs might cause a reduction in glutamate release at afferent terminals. Increasing the salience of the CS or US may cause sufficient afferent activation to overcome this autoreceptor action and permit normal learning. The idea that mGluRs modulate the signal to noise ratio in hippocampal dependent learning is discussed in section 3.12.5.

3.12.3 Anxiolytic actions of mGluRs

Before considering the role of mGluRs in spatial learning, it is worth noting that certain mGluR ligands have anxiolytic properties, a factor that may contribute to the apparent learning impairments seen after pharmacological manipulation of mGluR function. For instance, the novel group II mGluR agonist (+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY354740) has been reported to increase open arm activity in the elevated plus maze test, indicating a reduction in anxiety (Monn et al., 1997). It is postulated that this effect results from the suppression of excitatory pathways within the limbic system, consistent with the postulated autoreceptor actions of group II mGluRs. LY354740 has also been found to attenuate the enhancement of the auditory startle response following nicotine withdrawal in rats (Helton et al., 1997). Independent studies have demonstrated that antagonists of group I mGluRs ((*S*)-4C3HPG) and group III mGluRs (MSOP) reduce anxiety in rats, as measured using the conflict drinking Vogel test (Chojnacka-Wojcik et al., 1997). Since MCPG antagonizes group I and II mGluRs in expression systems, and has been reported to have agonist properties at group II mGluRs *in vivo*, application of MCPG might be expected to have anxiolytic effects.

However, MCPG has been shown to have little effect on the open field behaviour of rats, and the effects of MCPG and AIDA on shock-reinforced tasks do not indicate that these drugs have anxiolytic properties (Riedel et al., 1994a, 1995d; Nielsen et al., 1997). Nevertheless, the possibility that MCPG might have anxiolytic actions has never been directly tested, and the issue will be discussed further in section 6.5.

3.12.4 mGluRs and spatial learning

A number of studies have indicated that mGluRs are involved in various forms of hippocampus-dependent spatial learning. For example, watermaze studies involving mGluR knockout mice have revealed a spatial learning deficit in mice lacking mGluR1 (Conquet et al., 1994) and mGluR5 (Lu et al., 1997). However, most studies have involved pharmacological manipulations. Riedel et al. (1994a) found that an i.c.v. injection of MCPG prior to training in a foot-shock reinforced Y-maze spatial alternation task, resulted in a deficit when rats were later tested for retention. Paradoxically, the mGluR agonist *t*-ADA also caused a deficit when administered prior to training in the same task (Riedel et al., 1995b). However, application of *t*-ADA immediately post-training facilitated memory formation (Riedel et al., 1995c). Neither MCPG nor *t*-ADA had any effect on brightness discrimination learning. The differential effects of this putative group I mGluR agonist are interpreted by the authors according to the signal to noise ratio theory of mGluR function.

Using a slightly different approach Ohno and Watanabe (1996) found that blockade of mGluRs with MCPG could aggravate the working memory deficit in a three panel runway task resulting from NMDA receptor blockade. A subsequent study revealed that application of the selective group I mGluR antagonist AIDA alone could increase working memory errors in this task. However, concurrent administration of D-cycloserine, a partial agonist at the glycine site of the NMDA receptor, significantly reduced the impairment induced by AIDA (Ohno and Watanabe, 1998). These results suggest that mGluRs interact with NMDA receptors in memory formation: this interpretation is consistent with reports that group I mGluR activation can potentiate responses to NMDA (see section 3.7.4) and enhance tetanic LTP induced by a weak tetanus (see section 3.10.8). In Ohno and Watanabe's (1998) study, upregulation of NMDA receptors with D-cycloserine may bypass the necessity for mGluR activation, and allow normal learning in the presence of AIDA.

Curiously, application of AIDA has been reported to facilitate memory under certain circumstances. In a recent study study, the effects of AIDA were investigated in a delayed-matching-to-position task with fixed reward location in a three choice maze. Pre-training application of AIDA resulted in enhanced within session performance, but retention between sessions (separated by 24 hr) was equivalent to that of controls. This result differs from that obtained with a contextual fear conditioning task, in which AIDA caused only a slight and non-significant facilitation of within

session performance, but caused a pronounced retention deficit (G. Riedel, personal communication). These results suggest that blockade of group I mGluRs may have differential effects on short and long-term memory formation, manifested in a task-dependent fashion. Nevertheless, the reason for this dissociation is currently unknown.

The first investigation of the role of mGluRs in watermaze performance was published by Richter-Levin et al. (1994). A bilateral i.c.v. infusion of (*R*,*S*)-MCPG prior to training was found to cause little impairment in latency to reach the platform, but a severe deficit in transfer test performance. This deficit was observed regardless of whether MCPG was re-administered prior to the transfer test, ruling out the possibility of state-dependent or performance deficits. However, it has subsequently been reported that MCPG induces a slight increase in latency to find the hidden platform, but only causes a transfer test deficit when re-administered prior to testing; rats infused with MCPG throughout testing, but given saline prior to the transfer test performed as well as controls (Bordi et al., 1996). This result is hard to reconcile with that of the earlier study. Furthermore, in contrast to the positive results reported following acute i.c.v. injection of MCPG, chronic i.c.v. infusion of either MCPG or AIDA via an osmotic minipump does not disrupt watermaze performance (Riedel et al., 1998). These issues will be explored further in the experimental work described in chapter 6.

Application of mGluR agonists prior to training has also been reported to impair watermaze performance. Pettit et al. (1994) found that i.c.v. infusion of a subtoxic dose of (1*S*,3*R*)-ACPD increased latency to find the hidden platform, and resulted in a trend towards a transfer test deficit. Similar results have been obtained with L-CCG-I, although transfer test performance was unaffected (Van der Staay et al., 1995). However, the effective dose of this drug caused convulsions during infusion, post-infusion sedation, and increased floating in the pool. Hence, the "mnemonic" effects of this drug must be viewed with some caution. Hölscher et al. (1996) reported that i.c.v. infusion of L-AP4, an agonist at group III mGluRs, caused a deficit in both watermaze and radial maze tasks. Despite the fact that injection of the group III mGluR antagonist MAP4 alone caused a spatial learning deficit, co-administration of MAP4 and L-AP4 resulted in normal performance on both behavioural tasks. In addition to acting as a group III mGluR agonist, L-AP4 has also been found to antagonize the (1*S*,3*R*)-ACPD-induced increase in transmitter release thought to be mediated by an uncloned receptor (Herrero et al., 1994). Hölscher et al. (1996) have suggested that the effects of L-AP4 on spatial learning may be mediated by this mGluR subtype.

3.12.5 mGluRs may modulate the "signal to noise ratio" during learning

It is notable that whereas pre-training administration of mGluR agonists has been found to impair memory formation (Pettit et al., 1994; Riedel et al., 1995b), post-training application has been found to facilitate memory (Bianchin et al., 1994; Riedel et al, 1995c). These findings are interpreted by

Riedel (1996) as evidence that abnormal pre-training activation of mGluRs constitutes noise, and prevents efficient learning. It is suggested that mGluRs involved in a learning event may be use-dependently primed, leading to enhanced second messenger stimulation when activated by the application of an agonist. Hence, post-training mGluR agonist application may enhance memory formation since the greatest increases in signal transduction will be coupled to those mGluRs activated during the learning event, i.e. the signal to noise ratio will be increased.

Whilst this hypothesis is currently formulated in rather general terms, it provides a framework for the interpretation of a number of results, as well as generating testable predictions. However, a number of physiological mechanisms would be consistent with the signal-to-noise ratio hypothesis. For instance, "noise" could consist of a literal increase in spontaneous neuronal activity following mGluR activation (see Boddeke et al., 1997; Martin and Morris, 1997). However, a reduction in the statistical efficiency of a conjunction detection mechanism during excessive mGluR activation would also constitute the introduction of "noise" to a learning system. Unfortunately, these issues are difficult to address when the nature of the neuronal "signals" underlying learning are largely unknown.

3.12.6 Summary

The studies reviewed in this final section illustrate the diverse effects that the modulation of mGluR activity has been reported to have on behaviour. However, there is no overall consensus concerning the mechanisms by which mGluR blockade affects the performance of learning tasks, and the effects of an mGluR ligand on learning and its effects on LTP are often far from clear. A large part of the experimental work described in this thesis was conducted in an attempt to address these issues. This work is presented in chapters 6 and 7.

Chapter Four

General Methods

4.1 Subjects

Adult male Lister-hooded rats (250-500g) obtained from the breeding colony in the Department of Pharmacology, University of Edinburgh, were used as subjects in most experiments. However, differences in strain, sex or supplier will be mentioned as appropriate. Animals were individually housed with *ad libitum* access to food and water, and maintained on a 12 hr light / 12 hr dark cycle.

4.2 Watermaze training

4.2.1 Apparatus

Testing was carried out in an open-field watermaze (Morris 1981, 1984; figure 4.2), 2 m in diameter and 0.6 m deep, filled with water at 25 ± 1°C to a depth of 0.3 m. The pool was located in the centre of a room containing prominent extra-maze cues such as wall posters, a metal rack, cupboards etc. The room was diffusely illuminated by floodlights placed in the 4 corners. Rats were placed into the water facing the sidewalls and learned to escape onto a submerged platform, 11 cm in diameter. (On some occasions, however, a visible platform with prominent vertical black and white stripes protruding 1-1.5 cm above the water surface was used instead.) In order to obscure the submerged platform, the water was made opaque by the addition of cempolatex liquid (Cementone-Beaver Ltd.). A recessed video camera was placed directly above the centre of the pool to monitor the paths taken by the rats during swimming. The video signal was relayed to a video recorder for both on- and off-line analysis and fed to an image analyser (HVS VP112). Co-ordinates were sampled at 10 Hz by an Acorn Archimedes 3000 computer running the "Watermaze" software package written by R. Spooner. This program records the swim path of a rat, and calculates a number of behavioural measures, such as the latency to reach the escape platform and the percentage time spent within a specified area of the pool.

4.2.2 Procedure

Details of the behavioural methodology and data analysis differ between experiments, and will be described in subsequent chapters as necessary. However, the standard spatial reference memory task described below was used, with minor modifications, for all the experiments described in chapter 6.

4.2.2.1 Pre-training

Prior to spatial acquisition training, rats received one session of non-spatial pre-training consisting of 4 trials of 60 s each, during which curtains were drawn about the pool in order to obscure extra-maze cues. Both platform position and starting position were varied according to pseudo-random schedules. Rats which failed to find the platform within 60 s were guided to the correct location. A 30 s stay on the platform was allowed between each trial. This pre-training provided an introduction to the procedural aspects of the task, such as the need to avoid the side-walls and the existence of an escape platform, whilst keeping the spatial component to a minimum.

4.2.2.2 Acquisition training

For the following three days, rats were given daily blocks of 6 trials to a fixed platform location with the curtains drawn back to reveal extra-maze cues. Starting positions were randomized and animals that failed to find the platform within 120 s were guided to the correct location. Rats were allowed to remain on the platform for 30 s following the end of each trial, after which the next trial was started immediately. In order to control for positional biases, half of the rats were trained to a platform position in the centre of the NE quadrant of the pool, whilst the remainder were trained to a position in the SW quadrant. Platform positions were counterbalanced across all groups.

4.2.2.3 Transfer test

24 hr after the final acquisition training session, the rats' memory of the platform location was tested in a transfer test during which the platform was removed and the animal allowed to swim freely for 60 s. This test was analysed by dividing the pool into four quadrants centred around the NE, NW, SE and SW platform positions. The percentage time spent in each of these quadrants during the trial constituted the primary measure of task performance.

4.2.2.4 Visually cued task

At the end of training, rats received six visually cued trials that required the location of a black and white striped platform, protruding 1 cm above the water surface. Both platform and starting positions were randomized between trials and curtains were drawn about the pool in order to minimise interference from previously learned spatial information. Rats remained on the platform for 30 s between trials.

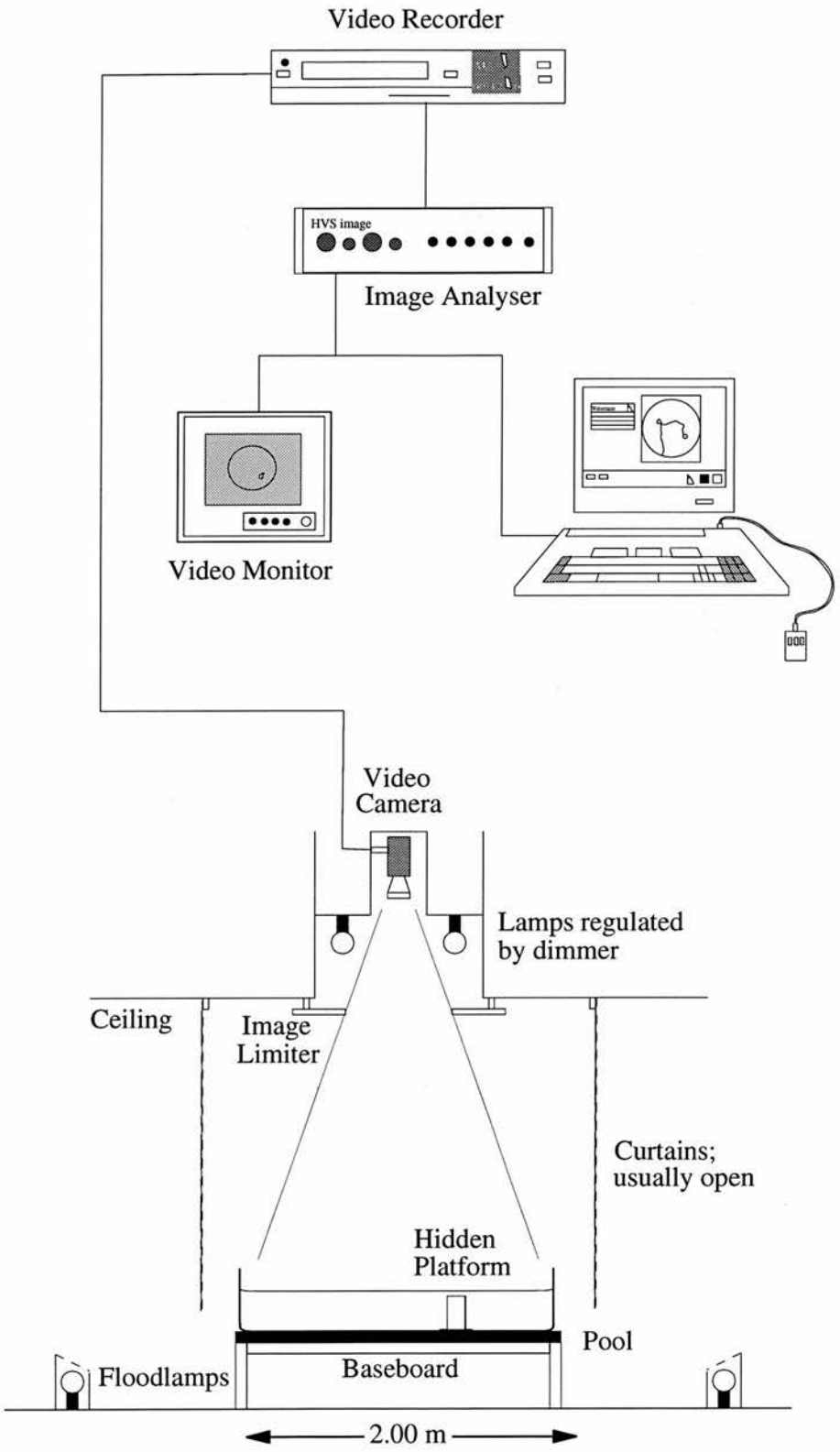


Fig. 4.2 Watermaze apparatus

4.3 Electrophysiology

Rats were anaesthetized with urethane (1.5g/kg i.p.), and mounted in a stereotaxic frame (Kopf) with skull horizontal. All co-ordinates described below, and in future chapters, were measured relative to bregma unless otherwise stated. Temperature was monitored by a rectal probe and maintained at 36.2 ± 0.2 °C using an isothermic heating blanket (Harvard Instruments). A teflon-coated, bipolar, platinum / iridium stimulating electrode (consisting of two wires twisted together, each wire having external diameter = 0.112 mm and bare wire diameter = 0.075 mm) was lowered into the angular bundle of the perforant path (AP = 7.5 mm; Lat. = 4.0 mm; DV ≃ 2.5 mm from the dura) and a monopolar recording electrode (a single wire of same diameter and material as the stimulation electrode) was positioned in the hilus of the dentate gyrus (AP = 3.5 mm, Lat. = 2.0 mm; DV ≃ 3.0 mm) in order to record positive-going field potentials.

Test stimulation was delivered using a NeuroLog stimulation system (Digitimer Research Instrumentation) at either 0.1 Hz or 0.05 Hz, and consisted of biphasic pulses with 100 μs half-pulse duration. Waveforms were amplified using a polygraph (Grass Instruments); the initial slope of the field EPSP (measured by linear regression between two fixed time points), and the population spike amplitude (the distance between the minimum of the negative-going spike and a tangent subtending the two local maxima of the field potential), were monitored on-line by an Acorn A5000 computer running specialized software. Deviations from this procedure, including the implantation of thermistors and / or infusion cannulae will be described in later methods sections.

After implantation of the electrodes, stimulation was turned off for at least half an hour in order to allow the brain tissue to settle. After this period a number of test pulses were delivered and the stimulation intensity was adjusted to elicit a population spike of the required amplitude. Several different sets of baseline stimulation parameters were used in the course of this thesis. Similarly, a number of different tetanization protocols were tested. These details are described wherever necessary.

4.4 Surgery for intracerebral drug delivery

4.4.1 Implantation of osmotic minipumps

Rats were placed in a stereotaxic frame (Kopf) under tribromoethanol anaesthesia (Avertin; 0.29 g/kg). The skull was exposed and a burr hole was made to allow the insertion of an L-shaped stainless steel cannula into the right lateral ventricle (co-ordinates from bregma: AP = 0.9 mm; Lat. = 1.3 mm; DV = 4.5 mm from skull surface). The cannula was attached to an osmotic minipump (Alzet 2002) via plastic tubing and secured in place with dental acrylic and three jeweller's screws. The minipump was inserted into a subcutaneous pocket above the shoulder blades. Alzet 2002 minipumps infuse continuously for 14 days at a rate of 0.5 µl / hr.

4.4.2 Implantation of acute i.c.v. infusion cannula

Animals were placed in a stereotaxic frame and anaesthetized with Avertin as above. The skull was exposed and a burr hole was made to allow the insertion of a stainless steel guide cannula (23 gauge) into the right lateral ventricle (co-ordinates from bregma: AP = 0.9 mm; Lat. = 1.3 mm; DV = 4.0 mm from skull surface). The cannula was fixed in place with dental cement and two jeweller's screws, one anterior and one posterior, provided further anchorage. After implantation, the guide cannula was blocked by the insertion of a stainless steel dummy cannula, protruding 0.5 mm below the tip of the guide cannula. Both guide and dummy cannulae were made in the laboratory from stainless steel tubing.

Prior to infusion, the dummy cannula was removed from the guide cannula and replaced by an injection needle of the same length connected, via plastic tubing, to a microsyringe. The rat was then given a 5 µl intraventricular infusion, over a period of 2 min. The injection needle was left in place for approximately 1 min after the end of infusion, to allow diffusion of the drug away from the injection site.

4.4.3 Implantation of intrahippocampal infusion cannulae

The methods used were similar to those described above. However, commercially available guide cannulae (23 gauge), dummy cannulae, and injection needles were used (Plastics One). Dummy cannulae consisted of a length of steel tubing attached to a plastic cap which could be screwed onto the guide cannula, resulting in a more effective barrier against infection than that provided by the dummy cannulae described above.

Guide cannulae were implanted bilaterally above the dorsal hippocampi (co-ordinates from bregma: AP = -4.5 mm; Lat. = ± 3.0 mm; DV = -3.0 mm from the dura) Four jeweller's screws were used to provide anchorage after cementing.

Prior to infusion, the dummy cannulae were removed from the guide cannulae and replaced by injection needles protruding approximately 0.2 mm below the base of the guide cannulae. These were connected, via plastic tubing, to microsyringes held in a syringe drive. A volume of 1 µl was infused into each hippocampus over a period of 5 min. The injection needles were left in place for approximately 1 min after the end of infusion.

4.5 Histology

After behavioural testing, rats implanted with electrodes, cannulae, or minipumps were either perfused transcardially with physiological saline followed by 10 % formol saline or sacrificed without perfusion. In both cases, the brains were removed and placed in formol saline solution. When adequately fixed, the relevant section of a brain was frozen, cut into 30 µm sections, and stained with cresyl violet. Sections were then examined under the light microscope to determine the position of the cannulae, and to assess the amount of tissue damage and / or infection. Other histological techniques will be detailed wherever relevant.

4.6 Statistics

Unless otherwise stated, statistical comparisons were carried out using analysis of variance (ANOVA). In ANOVA designs involving one between subject variable and one within subject variable, the effect of the between subject factor was sometimes calculated for just one level of the within subject factor (analysis of "simple effects"). For instance, in a watermaze experiment, the effect of drug group might be investigated on individual acquisition trials. Such an analysis was only carried out after a main effect of the between subject factor had been established. Conversely, the effect of the between subject variable was sometimes calculated for just one level of the between subject factor (i.e. for just one group). In the former case, the error term for the between subject factor

was calculated using the pooled error term, rather than using just one level, of the within subject variable. The number of degrees of freedom of this pooled error term was calculated using Satterwaite's approximation. In circumstances requiring multiple *post hoc* comparisons, Newman-Keuls individual pairwise comparisons were always used.

In cases where the distribution of values was suspected to deviate substantially from normality and / or the variance between experimental conditions was grossly non-homogeneous, a Shapiro-Wilk test for significant deviations from normality, and a Levene test for homogeneity of variance, were carried out. If necessary, a non-parametric analysis was then conducted.

Most data were analysed using "CLR ANOVA" and "SPSS" statistical packages. Correlation coefficients were calculated using "Cricket Graph" and "SPSS". Numerical values are stated as mean ± standard error throughout.

Chapter Five

Blockade of spatial learning with D-AP5: "performance" deficits and possible neurotoxicity

5.1 General Introduction

The application of NMDA receptor antagonists has consistently been found to impair the performance of rats in spatial learning tasks (see chapter 2.6.6). However, it is still not entirely clear that these drugs cause a pure memory impairment, rather than interfering with other behavioural processes. Sensory, motor, and / or motivational deficits following the administration of NMDA receptor antagonists may all impair spatial "learning". Distinguishing between genuine learning impairments and "performance" factors such as these is one of the central challenges of behavioural pharmacology. In addition, it has recently been suggested that the impairment observed after application of NMDA receptor antagonists might be secondary to the neurotoxic effects of these drugs, rather than the blockade of NMDA receptors. The following sections will provide a review of the relevant literature, followed by an experimental study designed to address some of these issues.

5.1.1 NMDA receptor antagonists cause "performance" deficits in learning tasks

NMDA receptors are widely distributed in the brain (Monaghan and Cotman, 1985); hence, any mode of drug administration other than local infusion into the target structure will lead to the blockade of NMDA receptors in a number of brain regions. Intraventricular drug infusion is preferable to systemic injection as a mode of drug delivery to the hippocampus. Moreover, it is necessary for the delivery of drugs that do not readily cross the blood-brain barrier, such as D-AP5. However, this route of administration results in a widespread distribution of the drug throughout the forebrain (Butcher et al., 1991; Davis et al., 1992).

The possibility of NMDA receptor blockade in structures such as the striatum and thalamus is a serious problem. For instance, there is evidence that NMDA receptors in the lateral geniculate nucleus (LGN) are involved in the normal relaying of visual information (Sillito et al., 1990), rather than synaptic plasticity *per se*. Indeed, i.c.v. infusion of the competitive NMDA receptor antagonists CPP and AP7 has been found to impair brightness discrimination in the rat (Tang and Ho, 1988). This result is potentially worrying considering the importance of vision in tasks such as the watermaze, in which rats must navigate towards a hidden platform using information provided by distal cues. A similar role of NMDA receptors in normal synaptic transmission of somatosensory information in the ventrobasal thalamus has been identified (Salt, 1986, 1987). Furthermore, it has been reported that infusion of the competitive NMDA receptor antagonist CPP into the thalamus antagonizes the nocioceptive responses of single neurons to noxious thermal stimuli in anaesthetized rats (Eaton and Salt, 1990). It is possible that a blockade of thalamic NMDA receptors following the i.c.v. infusion of an antagonist might result in a reduction in the aversive qualities of immersion in cold water, and

hence a reduced motivation to learn a watermaze task.

In addition to the possibility of sensory impairments, NMDA receptor antagonists have been reported to cause a range of motor deficits. Injection of AP5 into the spinal cord has been found to result in analgesia and locomotor paralysis of the hindquarters (Cahusac et al., 1986). Although such effects are unlikely after ventricular drug infusion, NMDA antagonists can also cause motor deficits by actions at higher brain centres, such as the thalamus (Klockgether et al., 1984). Both competitive and non-competitive NMDA receptor antagonists, including AP5, are frequently observed to cause ataxia, characterized by a slowing of the righting reflex, an abnormal gait, and a loss of balance (Koek et al., 1987; Leung and Desborough, 1988; Contreras et al., 1988; Hiramatsu et al., 1989). During watermaze training rats are often observed to fall off the platform whilst performing a "wet-dog shake" (Morris et al., 1986a; Morris 1989). As well as tiring the animals, this phenomenon may reduce the value of the platform as an escape from the aversive properties of immersion in water. Hence, drug-treated animals may be less motivated to search for the platform, and may look for other means of escape, for instance by swimming around the walls of the pool (Cain et al., 1996; R. G. M. Morris, R. J. Steele, S. J. Martin, and J. E. Bell, unpublished observations).

However, there may be other reasons for such maladaptive behavioural patterns. In addition to causing ataxia, non-competitive NMDA receptor antagonists such as dizocilpine (MK-801) produce stereotypical behaviours at comparatively low doses. These behaviours consist of stylized movement patterns such as head weaving, sniffing, and grooming (Koek et al., 1988; Tiedke et al., 1990). Such behaviour is also observed after treatment with competitive NMDA receptor antagonists including AP5 (Schmidt, 1986; Koek et al., 1987). It is likely that these effects are mediated by the blockade of striatal NMDA receptors. In fact, stereotypical behaviours can be induced by direct striatal infusions of D,L-AP5 (Schmidt, 1986). Rats given chronic i.c.v. minipump infusions of D-AP5 sometimes display stereotypical behaviours in the watermaze, such as swimming continuously around the perimeter of the pool (thigmotaxic swimming), or swimming in apparently aimless circles. Occasionally, AP5-treated rats are observed to "swim over" the escape platform owing to a failure to inhibit swimming movements upon contact. These swimming movements sometimes continue even after a rat has been removed from the pool. For a detailed description of such impairments, see Cain et al. (1996).

Consistent with the stereotypical behaviour patterns associated with the administration of NMDA receptor antagonists, MK-801 has been reported to cause perseverative arm entries in an 8-arm radial maze spatial alternation task (Holter et al., 1996), and perseverative lever pressing in an operant task (Cohn and Cory-Slechta, 1993). It is not clear whether non-competitive NMDA receptor antagonists also cause perseverative deficits. However, chronic minipump infusion of D,L-AP5 has been reported to cause a deficit in the "differential reinforcement of low rates" (DRL) task, in which rats must delay lever pressing in order to obtain a reward (Tonkiss et al., 1988). AP5-treated rats tended to respond

too early, a result interpreted as an impairment in temporary memory storage. Alternatively, however, this result may be an illustration of AP5-induced perseverative lever pressing similar to that observed after administration of MK-801.

In addition to the deficits described above, NMDA receptor antagonists have been found to have direct effects on motivation. For instance, application of AP5 increases open arm exploration time in the elevated plus maze test, indicating that the drug has anxiolytic actions (Dunn et al., 1989). A reduction in the anxiety experienced by a rat upon immersion in the watermaze may reduce the incentive to escape, and hence impair learning. It has been speculated that the AP5-induced watermaze impairment may result from such a motivational deficit (Keith and Rudy, 1990).

5.1.2 Attempts to control for NMDA antagonist-induced sensorimotor disturbances

A number of control tasks have been carried out in order to rule out the contribution of sensorimotor and motivational disturbances to watermaze performance. In the simplest of these paradigms, rats are simply required to locate a visible platform in the absence of distal cues. AP5 does not generally cause a deficit when tested in this task (Morris, 1989; Butcher et al., 1990; Steele and Morris, 1999; but see Cain et al., 1996). However, the task is clearly very easy: escape latencies of a few seconds are generally observed from the start of testing. Hence, a ceiling effect might obscure any subtle impairment. Nevertheless, in an earlier study, Morris et al. (1986a) carried out a visual discrimination task, in which rats had to discriminate between two randomly located but visually distinct platforms, only one of which would support the rat and offer refuge. No impairment was observed in AP5-treated rats, despite the obvious difficulty of the task (rats took between 40-120 trials to reach a criterion of 90 % correct in one 10-trial session). However, the demands of this task are very different to those of the spatial reference memory task, in which distal cues must be used. For instance, a slight visual impairment might be obscured by the fact that visual discrimination performance is likely to require a lower level of acuity than the spatial task.

Nevertheless, the recent report that D-AP5 causes a delay dependent impairment in a watermaze delayed matching-to-place task provides strong evidence that the AP5-induced impairment is not merely a sensorimotor side-effect, unrelated to learning (Steele and Morris, 1999; see chapter 2.6.6). If this were the case, performance would be equally affected at all delays.

5.1.3 The effects of training protocol on the severity of sensorimotor disturbances

Variations in experimental protocol between laboratories may contribute to differences of opinion concerning the contribution of sensorimotor disturbances to the watermaze deficit caused by NMDA

antagonists. For instance, massed trials result in greater sensorimotor disturbances under AP5 than spaced trials. Davis et al. (1992) gave rats 6 trials per day for 5 days; the performance of animals treated with AP5 tended to deteriorate over trials, suggesting that fatigue might exacerbate the drug-induced deficit. Consistent failure to find the platform during massed trials may also exacerbate stereotypical behaviour patterns such as circling the sidewalls. It was subsequently found that implantation of minipumps containing 15 mM D-AP5 failed to cause a deficit in a one trial per day watermaze paradigm (D. M. Bannerman, unpublished observations). This dose of AP5 results in a hippocampal concentration known to impair watermaze performance when massed trial protocols are used (e.g. Davis et al., 1992). Hence, under conditions that minimize sensorimotor impairments, a higher dose of AP5 may be needed to disrupt performance.

The sensorimotor disturbances induced by NMDA antagonists are strongly influenced by watermaze experience. The importance of prior experience in watermaze tasks is illustrated by the findings of Bannerman et al. (1995) and Saucier and Cain (1995) (see chapter 2.6.6). Similarly, the sensorimotor disturbances following AP5 infusion can be reduced by pre-training consisting of six trials to a visible platform with distal cues obscured (see Bannerman et al., 1995; experiment 1). Despite increasing in the course of a massed-trial training session, sensorimotor deficits associated with chronic AP5 infusion tend to diminish between testing days. AP5-treated rats are often found to fall off the platform on the first day of watermaze training, but this problem disappears in subsequent sessions. In a recent study conducted in this laboratory, the severity of a number of sensorimotor disturbances, both negative and positive, over several daily test sessions. By day 4, AP5-treated rats were indistinguishable from controls in terms of the behavioural symptoms of sensorimotor disturbance, yet they still showed a robust spatial learning deficit (R. G. M. Morris, R. J. Steele, S. J. Martin, and J. E. Bell, unpublished observations).

Nevertheless, it has recently been claimed that acute infusion of D-AP5 causes sensorimotor deficits in the watermaze that are severe enough to account for the failure to locate the platform (Cain et al., 1996). The disturbances reported include periphery swimming, contacting the platform without climbing on, "swimming" over the platform, and the continuation of hind-limb swimming movements after removal from the pool. Furthermore, AP5-treated rats swam more slowly relative to controls as testing progressed, and were impaired in the performance of a visually cued control task. However, rats were given a single session of hidden platform training, consisting of 10 trials in close succession, followed by 10 trials to a visible platform. This is a protocol likely to exacerbate sensorimotor disturbances, as well as inducing considerable fatigue in animals failing to find the platform rapidly. This may explain the impairment found in the visible platform task, a result that has not been obtained in other studies (Morris, 1989; Butcher et al., 1990). Furthermore, it is not clear whether sensorimotor disturbances were evident from the start of testing, or whether they became worse as testing progressed, perhaps as the result of a failure to learn. In order to investigate these issues, it was decided to repeat the study presented by Cain and colleagues, subject to minor

modifications (see experiment 5.2).

5. 1. 4 Neurotoxic effects of NMDA receptor antagonists

In addition to the contribution of sensorimotor disturbances, Cain et al. (1996) suggested that the behavioural deficit caused by chronic infusion of NMDA receptor antagonists might be secondary to the neurotoxic actions of these drugs in the retrosplenial and cingulate cortices, brain regions known to be involved in spatial learning (Sutherland et al., 1988).

The neuroprotective effects of NMDA receptor antagonists following insults such as hypoxia and ischaemia are well established, and have been the subject of considerable clinical interest. It is known that the high synaptic concentrations of glutamate which follow a number of insults are excitotoxic, probably owing to excessive calcium influx via NMDA receptors (see Bruno et al., 1993; Choi, 1992 for review). Application of non-competitive and competitive NMDA receptor antagonists, including D,L-AP5 has been found to protect neurons in rat hippocampal slices from hypoxic damage (Grigg and Anderson, 1990). However, most research has been carried out with the non-competitive antagonists dizocilpine (MK-801), phencyclidine (PCP) and ketamine. The most potent of these compounds, MK-801 has been found to prevent excitotoxic neuronal damage in animal models of ischaemia (Gill et al., 1987) and epilepsy (Clifford et al., 1990). However, non-competitive NMDA receptor antagonists have clinically undesirable psychotomimetic properties. In addition, these drugs have recently been discovered to be neurotoxic themselves.

Olney et al. (1989) found that subcutaneous injection of relatively low doses MK-801, PCP, or ketamine in female Sprague-Dawley rats caused cytoplasmic vacuolation in neurons of the retrosplenial and posterior cingulate cortices. This reaction first became evident 2 hr after injection, and became increasingly more prominent at 4, 8, and 12 hr. However, 24 hr after drug administration, most neurons appeared normal. A similar acute vacuolation was obtained following injection of D-AP5 into a site bordering on the cingulate cortex (Labruyere et al., 1989). A number of other competitive NMDA receptor antagonists have since been found to induce the same reaction (Hargreaves et al., 1993; Ellison, 1994). In addition to vacuolation, injured neurons of the retrosplenial cortex have been found to express a 72k Da heat shock protein (HSP72). This response was maximal 24 hr after treatment with MK-801, but was still detectable for up to 2 weeks (Sharp et al., 1991; Lan et al., 1997).

Despite the fact that vacuolation was initially thought to be reversible, subsequent studies have demonstrated that higher doses of MK-801 can cause necrosis in the retrosplenial cortex, evident several days after a single drug treatment (Allen and Iversen, 1990; Fix et al., 1993). Curiously, female rats are found to be more susceptible to neuronal damage than males (Fix et al., 1995). It has

been suggested that after doses of MK-801, vacuolated neurons may become irreversibly injured, leading to necrosis (Fix et al., 1993). However, the relationship between vacuolation, HSP72 expression and necrosis is currently unclear.

Application of NMDA receptor antagonists for an extended period has been reported to cause a more widespread pattern of neuronal damage. Repeated administration of MK-801 (i.p. injection every 8 hr for 2 days) resulted in neuronal degeneration in the pyriform cortex, entorhinal cortex, amygdala, and the ventral two thirds of the dentate gyrus, as well as the cingulate and retrosplenial cortices (Horváth et al., 1997). Damaged neurons, axon terminals and activated microglia were labelled using silver staining techniques. A similar, but less severe pattern of damage was obtained following single high doses of MK-801. In most brain regions, the highest density of silver impregnation was observed within the first day or two after injection. An extended pattern of degeneration has also been found following the chronic administration of PCP by osmotic minipump (Ellison and Switzer 1993). Chronic minipump infusion of the competitive NMDA receptor antagonist LY235959 has been reported to induce a similar pattern of limbic degeneration, as revealed by silver staining techniques, to that induced by non-competitive antagonists (Ellison, 1994).

The mechanism by which NMDA receptor antagonists cause neuronal damage is unclear. Olney et al. (1991) proposed that glutamatergic neurons in the posterior cingulate cortex give rise to collateral axons which feed back to NMDA receptors on GABAergic neurons and exert tonic inhibitory control over the release of acetylcholine onto cingulate neurons. The disinhibition of cholinergic afferents following NMDA receptor blockade thus leads to neurotoxic damage. This scheme is consistent with reports that the neurotoxic effects of MK-801 can be attenuated by application of the cholinergic antagonist, scopolamine (Olney et al., 1991; Wozniak et al., 1995), or by anaesthetic agents which facilitate $GABA_A$ receptor-mediated neurotransmission (Olney et al., 1991; Ishimaru et al., 1995; Jevtovic-Todorovic et al., 1997). However, it is possible that disinhibition of other excitatory pathways may be involved (Corso et al., 1997). It has recently been reported that the protein synthesis inhibitor cycloheximide almost completely prevents neuronal damage caused by injection of MK-801 (Zhang et al., 1996). This suggests that the neuronal death caused by NMDA receptor antagonists may be apoptotic, rather than necrotic, as had previously been assumed.

Administration of MK-801 has been reported to cause an acute impairment in a number of spatial learning tasks, attributed to a deficit in memory function (Butelman, 1989; Caramanos and Shapiro, 1994; Pitkanen et al., 1995). However, the potential neurotoxic effects of the drug have rarely been considered. In a recent study by Wozniak et al. (1996), mice given a single i.p. injection of a high dose of MK-801 were impaired in the performance of a hole board food search task for a period up to at least 5 months after treatment. Mice injected with a lower dose of MK-801 showed only an acute impairment. The performance of high-dose MK-801-injected mice did not differ from that of untreated animals in a range of sensorimotor control tasks carried out 2 weeks after injection.

Histological analysis confirmed that treatment with high-dose MK-801 resulted in the death of a small number of neurons in the cingulate and retrosplenial cortices. These results demonstrate that an acute dose of an NMDA receptor antagonist can lead to a chronic spatial learning impairment, probably due to drug-induced neuronal death.

However, the possibility of neuronal death following chronic i.c.v. infusion of the competitive NMDA receptor antagonist D-AP5 has never been investigated. Consequently, the purpose of experiment 5.3 was to investigate the possible neurotoxic effects of i.c.v. minipump infusion of D-AP5, in comparison to the effects of acute systemic injections of MK-801 which reliably cause neuronal damage. In addition, the spatial learning abilities of rats were assessed both during and after the period of AP5 infusion (experiment 5.2), in order to determine the contribution of chronic neuronal damage, if found, to the AP5-induced behavioural deficit.

5.2 Chronic administration of D-AP5 impairs performance in a spatial, but not a cued version of the watermaze task, only during the period of drug infusion

5.2.1 Introduction

The purpose of this study was to replicate the behavioural protocol used by Cain et al. (1996), subject to minor modifications. Owing to the suggestion that AP5 infusion might cause brain damage, different groups of rats were tested either during or after the period of drug infusion. The latter group was included in order to investigate the possibility of persistent deficits attributable to AP5-induced neuronal damage.

An additional aim of this experiment was to assess whether sensorimotor disturbances might be exacerbated by the testing protocol used by Cain et al. (1996), in comparison to the protocols routinely used in this laboratory (see section 5.1). Of particular interest was the question of whether the visible platform deficit reported by Cain and colleagues could be obtained using chronic i.c.v. minipump infusions of D-AP5.

5.2.2 Methods

5.2.2.1 Animals

Adult male Lister-hooded rats (250-500g) obtained from the breeding colony in the Department of Pharmacology, University of Edinburgh, were used as subjects.

5.2.2.2 Drugs

All drug solutions were made up in pyrogen free water. A 100 mM stock solution of D-2-amino-5-phosphopentanoate (D-AP5; Tocris) was made up in equimolar NaOH. A final concentration of 30 mM D-AP5 was attained by dilution with the appropriate volume of artificial cerebrospinal fluid (aCSF), made up according to the Alza methodology (final ionic concentrations (mM): Na^+ = 150.0; K^+ = 3.0; Ca^{2+} = 1.4; Mg^{2+} = 0.8; PO_4^{3-} = 1.0; Cl^- = 155.0; pH = 7.3 ± 0.1). Solutions were stored at -20°C in small aliquots before use.

5.2.2.3 Surgery

Since D-AP5 does not readily cross the blood-brain barrier, AP5 was infused directly into the brain

via a surgically implanted osmotic minipump (see chapter 4.4.1 for details). 13 rats received minipumps filled with 30 mM D-AP5; a further 12 received aCSF. The Alza 2002 minipump infuses continuously at 0.5 µl / hr for 14 days, after which the pump is exhausted.

5.2.2.4 Behavioural testing

Testing was carried out in an open-field watermaze, as described in chapter 4.2.1. After surgery, rats were divided into 2 groups. In one group, "pumps on", behavioural testing was carried out during the period of drug infusion, starting 5 days after minipump implantation (30 mM D-AP5: n = 7; aCSF: n = 6). In the second group, "pumps off", testing was carried out after exhaustion of the minipumps, starting 25 days after implantation (30 mM D-AP5: n = 6; aCSF: n = 6). Both groups received identical watermaze training. All rats were given pre-training one day before testing was started, consisting of 6 trials to a totally random platform location with curtains drawn about the pool to obscure extra-maze cues. Trials lasted for a maximum of 1 min each; rats which failed to find the platform within this time were guided to the correct location. Animals were allowed to remain on the platform for 30s following each trial, after which the next trial was begun immediately.

The main phase of watermaze training followed a protocol similar to that described by Cain et al. (1996). On day 1, rats were given spatial training consisting of a 60 s "transfer test" (escape platform absent), then 10 trials to a fixed hidden platform position in 5 blocks of 2 trials each (inter-trial interval = 2 min; inter-block interval = 5 min), and finally a second 60 s transfer test to assess the rats' memory for the position of the escape platform. During transfer tests, rats were simply allowed to swim freely for 60s with the escape platform absent. These trials were analysed by dividing the pool into 4 quadrants, one of which, the "training quadrant", was centred about the position of the now absent platform. The percentage time spent within this quadrant provides a more sensitive and less variable measure of memory for the platform position than latency scores alone. A 5 min interval separated each transfer test from the regular training trials. Starting positions were moved pseudo-randomly between trials (N, S, E or W). The goal platform was located either in the centre of the NE or the SW quadrant of the pool. These positions were counterbalanced across groups in order to control for positional biases. Rats were allowed to remain on the platform for 15s after completion of each training trial.

On the following day, rats were given a visually cued task (in contrast to the Cain et al. study in which both spatial and cued tasks were carried out on the same day). Testing consisted of 10 trials (5 blocks of 2) to a visible, striped platform, 10 cm in diameter and protruding 1-1.5 cm above the water surface. White curtains were drawn about the pool to obscure extra-maze cues. Rats were always placed in the water at N, and the platform was moved pseudo-randomly between each trial to 1 of 4 locations (N, S, E and W). Rats were allowed to remain on the platform for 15 s after each trial, and the schedule of inter-trial intervals was identical to that described for the hidden platform training.

5.2.2.5 Histology

At the end of training, rats were injected i.p. with Euthatal (sodium pentobarbitone; 1 ml) and perfused transcardially with physiological saline followed by 10 % formol saline. Brains were then removed, and a coronal wedge of tissue was taken from the region immediately adjacent to the cannula site. This section was placed in formol saline solution. The rest of the brain was preserved for later histological investigation of the possible neurotoxic effects of AP5 infusion (see experiment 5.3). However, the wedge of tissue surrounding the cannula site was frozen, cut into 30 μM sections, and stained with cresyl violet in order to determine the cannula position and to assess any mechanical damage and / or infection caused by cannula implantation.

5.2.3 Results

AP5-treated animals trained in the spatial task during the period of drug infusion were grossly impaired relative to controls, and showed severe sensorimotor deficits which became progressively worse as training continued. AP5-treated animals in the "pumps off" condition displayed sensorimotor deficits such as impairment of the righting reflex during the period of AP5 infusion, but when tested after exhaustion of the minipumps, these rats performed as well as aCSF-treated controls, with minimal sensorimotor impairments.

5.2.3.1 Escape latencies during hidden platform training

Latencies to reach the escape platform in the spatial task are shown in figure 5.2.1. An ANOVA of performance in the "pumps on" condition (figure 5.2.1A), in which all trials were entered as within subject factors, revealed a significant overall improvement across trials [$F(9,99) = 2.07$; $p < 0.05$], and also a highly significant group difference, indicating that AP5-treated animals were impaired [$F(1,11) = 104.32$; $p < 0.0001$]. A significant drug group x trial interaction was found [$F(9,99) = 5.74$; $p < 0.0001$]. Subsequent analysis of simple effects revealed that AP5 treated animals showed no significant change in escape latency over trials [$F(9,99) = 1.70$; $p > 0.05$], compared to controls whose improvement was highly significant [$F(9,99) = 5.79$; $p < 0.001$]. No impairment was seen on trial 1, at which point neither group had any knowledge of the platform's location [Mean aCSF latency = 48.5 ± 5.5 s; mean AP5 latency = 40.0 ± 8.1 s; $F(1,94) = 1.26$; $p > 0.2$]. This result is consistent with the notion, discussed in depth later, that sensorimotor disturbances are initially minimal, but develop as training progresses.

However, on a substantial number of trials, AP5-treated rats failed to reach the platform within the maximum allowed time of 60 s. Owing to this, it was suspected that the data were likely to violate the

criteria for parametric analysis. A Shapiro-Wilk test for normality and A Levene test for homogeneity of variance were carried out. The tests revealed that the data failed to meet either of these criteria for parametric analysis, and so a non-parametric Kruskal-Wallis test was performed instead. This test revealed that AP5-treated animals showed equivalent escape latencies to controls on trial 1 [*Chi-square* = 0.68; $p > 0.4$], but were impaired on all subsequent trials [trials 2 and 8: *Chi-Square* > 4.0; $p < 0.05$; trials 3,4,5,6,7,9,10: *Chi-Square* > 7; $p < 0.01$].

An ANOVA of performance in the "pumps off" condition (figure 5.2.1B) did not reveal an overall group difference [$F < 1$]. However, the overall decrease in escape latencies over trials failed to reach significance [$F(9,90) = 1.75$; $0.05 < p < 0.1$]. In fact, performance of both AP5 and aCSF-treated rats in the "pumps off" condition was noticeably worse than that of aCSF-treated rats in the "pumps on" condition. A separate ANOVA of the aCSF groups from each condition only revealed a significant impairment in the "pumps off" animals [$F(1,10) = 5.91$; $p < 0.05$]. The reason for this impairment is unknown, although it is possible that the presence of an intracerebroventricular cannula for 25 days prior to testing may have impaired performance in some way. Nevertheless, transfer test performance was unaffected, as described below.

5.2.3.2 Transfer test performance

Figure 5.2.2A shows the performance of rats in the "pumps on" condition in transfer tests given before and after the 10 acquisition trials. The graph displays percentage time spent in the quadrant of the pool in which the platform was located during acquisition training (the "training" quadrant). AP5-treated rats performed at, or slightly below chance, both before and after training. However, aCSF-treated rats improved from 18.5 ± 3.1 % initially, to 48.9 ± 3.7 % after training. An ANOVA of percentage time within the training quadrant, in which each transfer test was entered as a within subject factor, revealed a significant overall effect of group [$F(1,11) = 12.12$; $p < 0.01$], and a significant group by transfer test interaction [$F(1,11) = 30.28$; $p < 0.001$].

Conversely, both AP5 and aCSF-treated rats showed an equivalent improvement between the first and second transfer test when trained after exhaustion of minipumps (figure 5.2.2B). Performance on the second transfer test was well above chance in both groups (percentage time in training quadrant: aCSF = 50.0 ± 5.4 %; AP5 = 43.1 ± 7.2 %). An ANOVA of percentage time in the training quadrant in pre- and post-training transfer tests (entered as within subject factors) showed neither a group difference [$F < 1$] nor a group x trial interaction [$F(1,10) = 1.19$; $p > 0.3$].

aCSF-treated rats in the "pumps off" condition performed worse than aCSF-treated rats in the "pumps on" condition in terms of latency to reach the platform during acquisition training. However, a separate ANOVA comparing aCSF performance on the post-training transfer test across the two

conditions, revealed no group difference [% time in training quadrant: aCSF, "pumps off" = 50.0 ± 5.4 %; aCSF, "pumps on" = 48.9 ± 3.7 %; $F < 1$].

5.2.3.3 Sensorimotor disturbances

The above analysis reveals that rats infused with 30 mM D-AP5 are severely impaired in the acquisition of a spatial reference memory task, but only during the period of drug infusion. However, sensorimotor disturbances may have contributed significantly to this impairment. During training, AP5-treated rats displayed many of the deficits noted by Cain et al. (1996), such as swimming around the periphery of the pool, "swimming" over the platform rather than climbing on, making contact with the platform without climbing on, loss of balance whilst standing on the platform (especially after a "wet-dog" shake), and in extreme cases, continuing to make swimming movements after removal from the pool. It was apparent that the AP5-treated rats showed an increase in all of these indices of impairment as testing progressed. A number of such sensorimotor impairments have been investigated in detail with the aid of a side-mounted video camera in a later experiment conducted in this laboratory (R. G. M. Morris, R. J. Steele, S. J. Martin, and J. E. Bell, unpublished observations). However, in the present study, only two indices of sensorimotor disturbance could be satisfactorily analysed: percentage time spent within 15 cm of the pool wall (an index of thigmotaxis), and mean swim speed.

5.2.3.4 Thigmotaxis

Figure 5.2.3A shows the time spent within 15 cm of the sidewalls during all trials. Data from the transfer tests are plotted together with regular training trials. An ANOVA in which all trials were entered as within subject factors revealed a significant main effect of trial [$F(11,121) = 3.39$; $p < 0.001$], a significant group difference [$F(1,11) = 37.90$; $p < 0.001$], and a significant group x trial interaction [$F(11,121) = 8.10$; $p < 0.0001$]. Analysis of simple effects showed that the trend towards a decrease in percentage time within 15 cm of the sidewalls in the aCSF group, from greater than 20 % on trial 1, to a stable value of less than 10 % from trial 4 onwards, was not significant [$F < 1$]. In contrast, AP5-treated rats showed a highly significant progressive increase in thigmotaxis over trials [$F(11,121) = 11.32$; $p < 0.001$]. However, a group difference in thigmotaxis did not emerge until the third acquisition trial [$F(1,101) = 4.17$; $p < 0.05$; ANOVA, simple effects], at which point the search strategy of AP5-treated animals abruptly began to deteriorate. Note, however, that on trial 2 of acquisition training, AP5-treated rats were no more thigmotaxic than controls, but took more than twice as long to find the platform (see figure 5.2.1). This result illustrates the fact that thigmotaxis is not a necessary consequence of AP5 infusion, but that it develops after repeated trials.

Figure 5.2.3B shows the percentage time within 15 cm of the sidewalls after exhaustion of the minipumps. A significant main effect of trial was found [$F(11,110) = 3.03$; $p < 0.01$], but no overall group difference was revealed [$F(1,10) = 1.90$; $p > 0.1$].

Figure 5.2.4 shows representative sample swim paths of rats in the "pumps on" condition. The rat infused with aCSF searched randomly during the first transfer test, but after training it spent approximately half its time within the training quadrant (figure 5.2.4A). The first AP5-treated rat shown (little thigmotaxis) simply searched randomly during both transfer tests, indicating that no learning had occurred (figure 5.2.4B). The paths taken by the second AP5-treated rat (severe thigmotaxis) were perhaps more typical (figure 5.2.4C). In this case, searching was random on the first transfer test, but thigmotaxic swimming was not evident at this point. By the second transfer test, however, swimming was limited almost completely to the periphery of the pool.

Figures 5.2.5A and B show examples of transfer test performance in the "pumps off" condition. Both aCSF and AP5-treated rats searched randomly during the first transfer test, but showed a strong bias towards the platform position after training.

5.2.3.5 Swim speed

Figure 5.2.6A shows swim speeds on all trials, including transfer tests, in the "pumps on" condition. An ANOVA revealed a significant main effect of trial [$F(11,121) = 3.48$; $p < 0.001$], a significant overall group difference [$F(1,11) = 9.03$; $p < 0.02$], and a significant group x trial interaction [$F(11,121) = 4.53$; $p < 0.0001$]. Analysis of simple effects revealed that AP5-infused rats slowed significantly as testing progressed [$F(11,121) = 7.73$; $p < 0.001$], whilst no change in swim speed was seen in the aCSF-infused group [$F < 1$]. Note that both groups swam equally fast during their first exposure to the pool on the first transfer test [simple effects: $F < 1$]. Apart from an anomalous point on the first acquisition trial, the difference in swim speeds did not become significant until trial 8 [simple effects: $F(1,46) = 12.07$; $p < 0.01$]. However, the deficit in escape latency was apparent from trial 2 onwards, well before swim speed began to fall. This pattern of results is similar to that found during the analysis of thigmotaxis, i.e. latency to find the platform was impaired *before* sensorimotor disturbances became evident.

Nevertheless, swim speed tends to fall gradually in the course of an individual trial, as rats become fatigued. It might therefore be expected that the longer the escape latency on a given trial, the lower the mean swim speed would be. Hence, it could be argued that the apparent group difference in swim speeds between AP5-treated rats and controls is simply an artifact of the difference in escape latencies. This is unlikely for a number of reasons. Firstly, escape latencies did not change over trials in the AP5 group, yet swim speeds were still found to decrease. Secondly, as mentioned above, the

group difference in swim speeds is not apparent until trial 8, whereas latencies are impaired from as early as trial 2. Thirdly, swim speeds differ considerably on the second transfer test, during which all rats swim for exactly 60 s. Hence, it is probable that AP5-treated rats simply became fatigued over the course of multiple trials lasting close to 60 s.

Figure 5.2.6B shows swim speeds for the "pumps off" condition. There was a significant main effect of trial [$F(11,110) = 2.44$; $p < 0.01$], but no significant group difference [$F < 1$].

5.2.3.6 Visually cued task

Figure 5.2.7 shows escape latencies during the visible platform task. Latencies were considerably shorter than those seen in the hidden platform task, and rats from all groups swam straight towards the platform on most trials. Significant main effects of trial were obtained in both "pumps on" and "pumps off" conditions [$F(9,99) = 5.37$; $p < 0.0001$; and $F(9,90) = 5.07$; $p < 0.0001$ respectively], but no group differences were found in either the "pumps on" [$F(1,11) = 1.17$; $p > 0.3$] or "pumps off" condition [$F(1,10) = 3.93$; $p > 0.05$].

The absence of an impairment in the AP5 "pumps on" group was particularly striking considering the gross impairment observed in the hidden platform task. AP5-treated rats displayed greatly reduced sensorimotor disturbances during the cued task, and rarely fell off the platform, even during "wet-dog" shakes. Figure 5.2.8 shows thigmotaxic swimming during visible platform testing. In the "pumps on" condition, there was a significant main effect of trial [figure 5.2.8A; $F(9,99) = 8.01$; $p < 0.0001$], but no significant overall group difference was found [$F(1,11) = 1.91$; $p > 0.1$].

Note that in accordance with the protocol described by Cain et al. (1996), the same sequence of platform positions was used in all rats, and N was always used as the starting point. Hence, trials in which the platform was located in the centre of the N quadrant, such as trial 4, necessarily generate high "thigmotaxis" values, especially if performance is optimal. It is significant that AP5-treated rats reached the visible platform in under 3 s on trial 4, faster than controls. It is therefore not surprising that a large proportion of this time was spent close to the sidewalls. The "thigmotaxis" peak on trial 6 likewise corresponds to a target location in the N quadrant.

In the "pumps off" condition (figure 5.2.8B), there was a significant main effect of trial [$F(9,90) = 5.04$; $p < 0.0001$], but no significant group difference in thigmotaxis [$F < 1$].

Figure 5.2.9 shows the swim speeds of rats during the visible platform task. A significant main effect of trial was obtained in the "pumps on" condition [figure 5.2.9A; $F(9,99) = 8.94$; $p < 0.0001$], but no group difference [$F(1,11) = 1.60$; $p > 0.2$]. In the "pumps off" condition, the main effect of trial was likewise significant [figure 5.2.9B; $F(9,90) = 3.97$; $p < 0.001$], but no significant group difference

was found [$F < 1$].

5.2.3.7 Histology

The infusion cannula was correctly sited in the right lateral ventricle in all cases. Little or no evidence of ventricular enlargement and / or brain infection was observed in any of the brains examined.

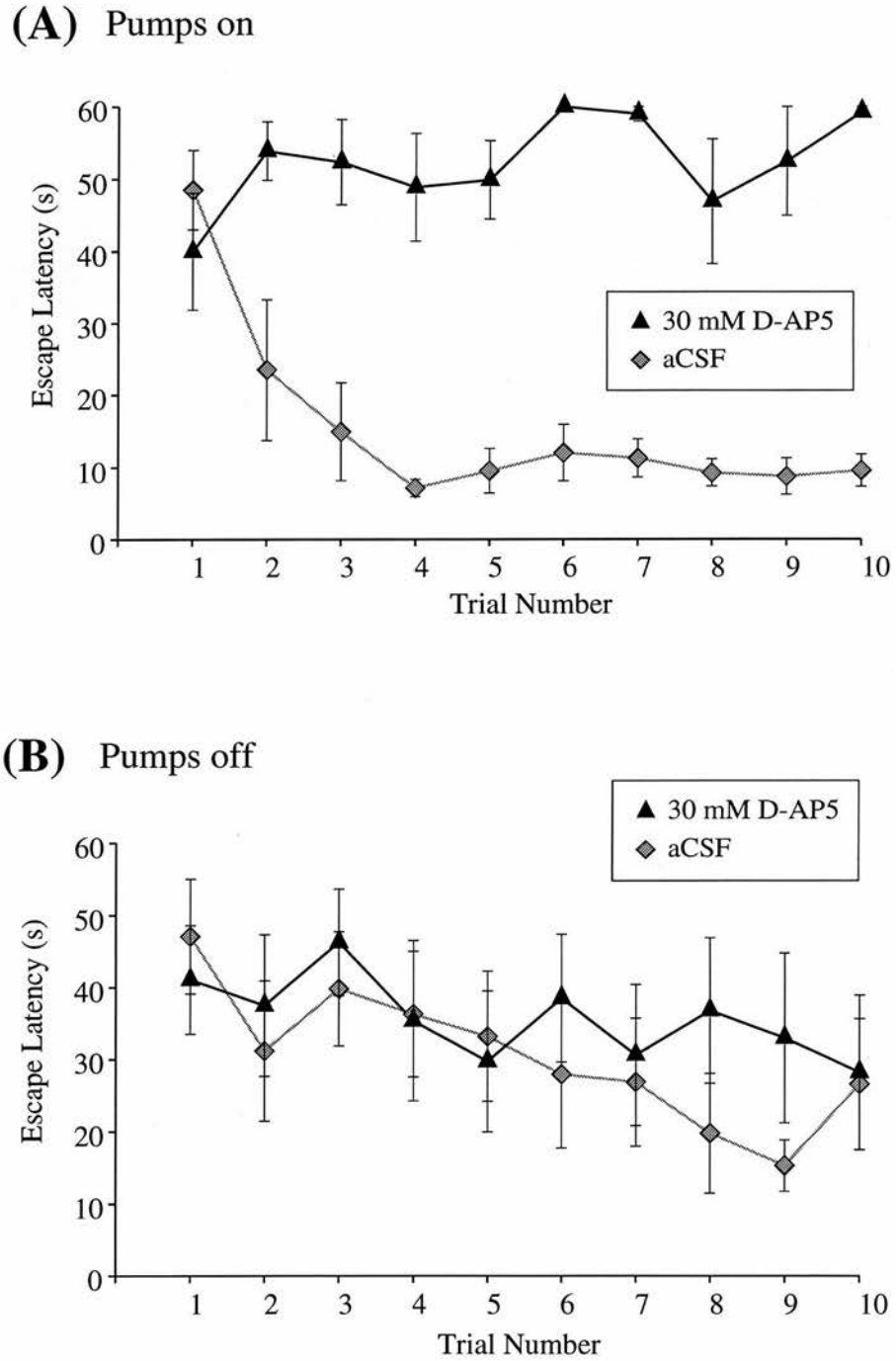


Fig. 5.2.1

(A) Minipump infusion of 30 mM D-AP5 impairs acquisition of a spatial reference memory task in the watermaze. (B) No such impairment is seen if rats are tested after the minipumps are exhausted.

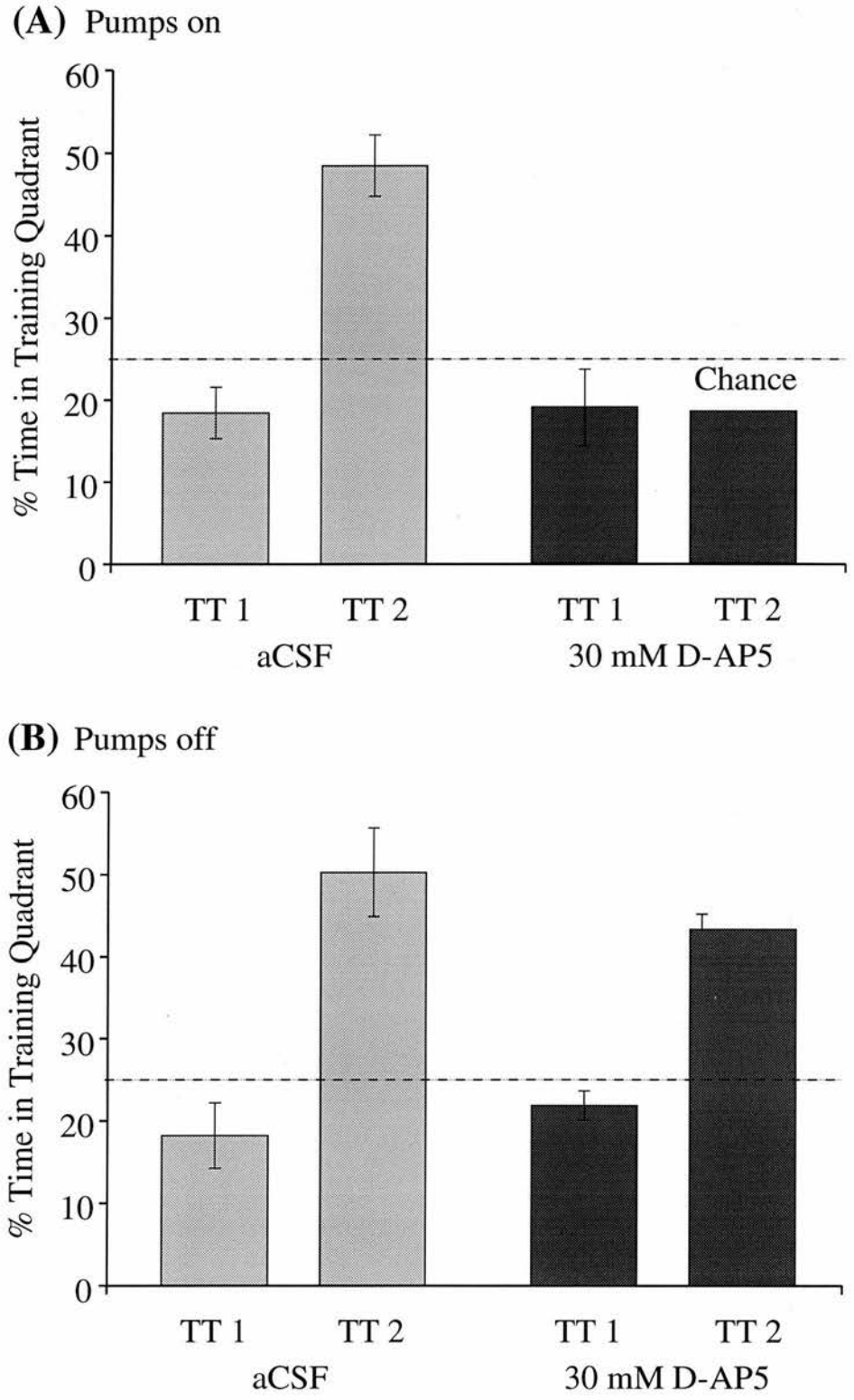
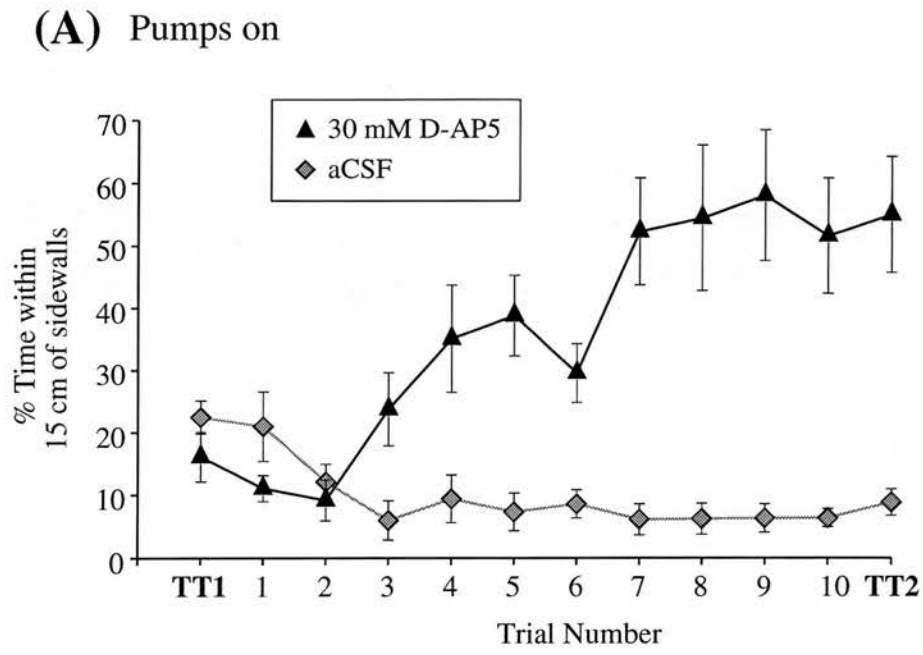


Fig. 5.2.2

Percentage time spent in the training quadrant during transfer tests given before (TT1) and after (TT2) spatial acquisition training. AP5 impaired performance during the period of drug infusion only.



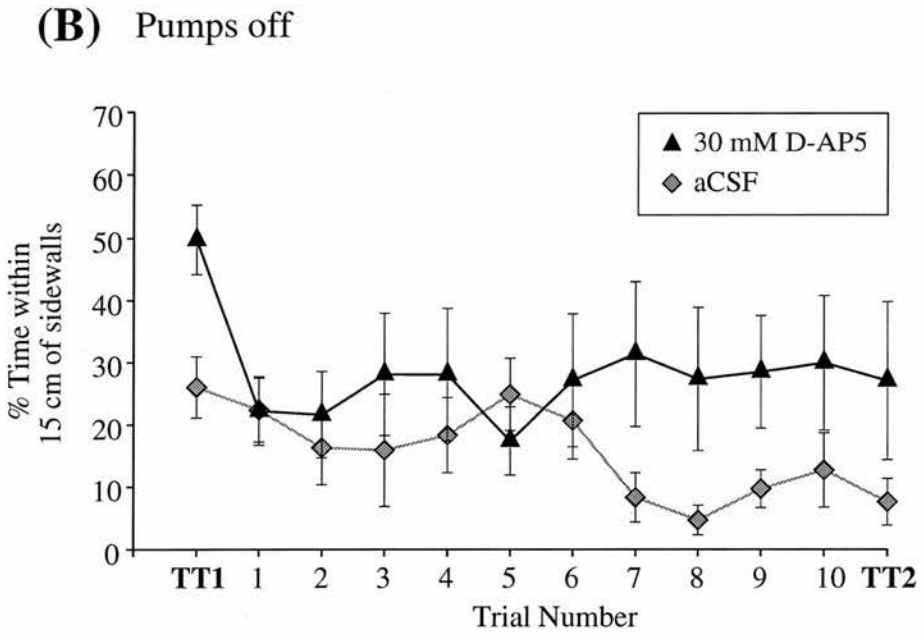


Fig. 5.2.3

(A) Percentage time spent within 15 cm of the sidewalls during each trial. Rats infused infused with 30 mM D-AP5 become progressively more thigmotaxic as testing continues. Data from transfer tests (TT1 and TT2) are plotted together with regular trials. (B) No group difference in thigmotaxis is seen when rats are tested after exhaustion of minipumps.

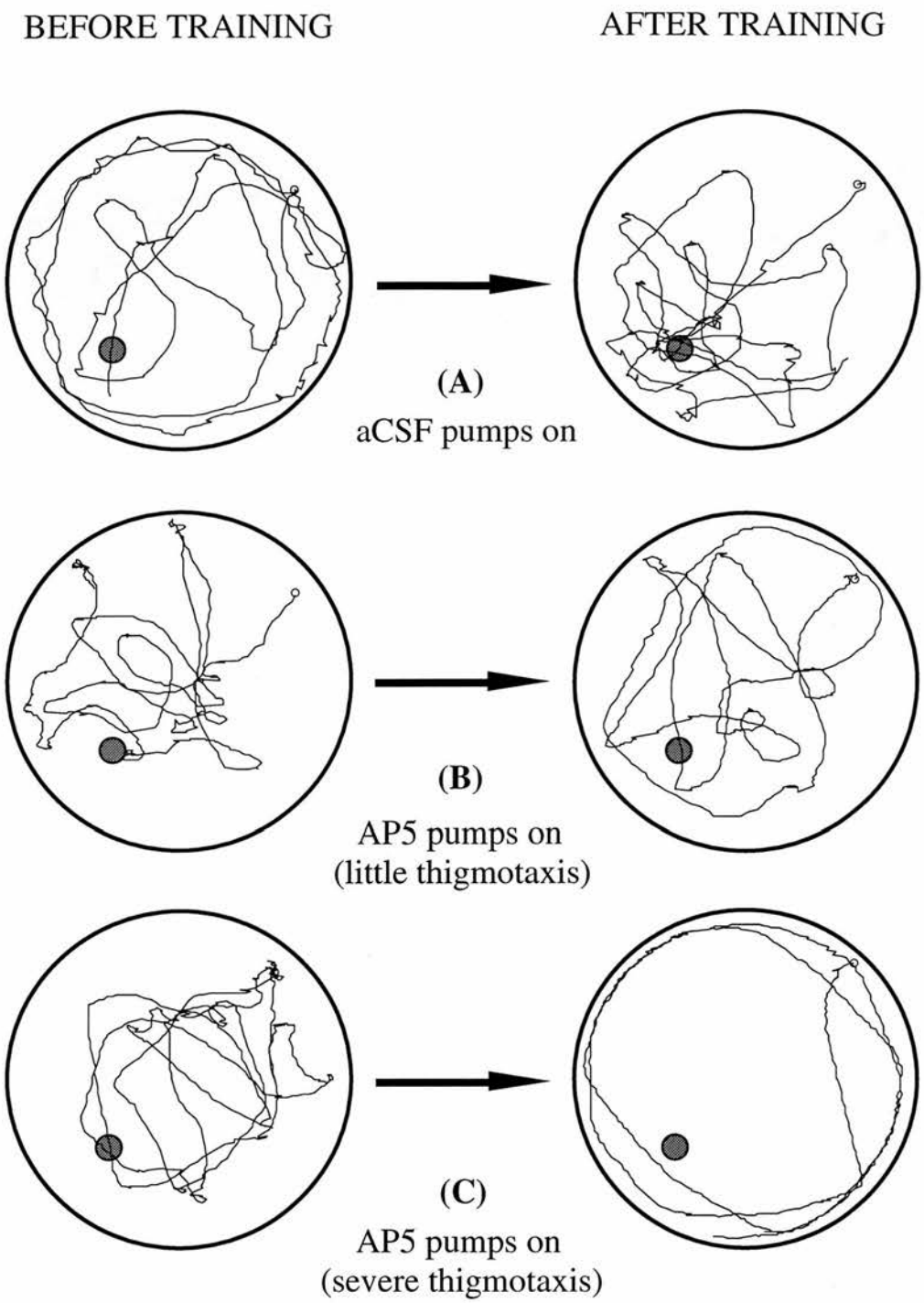


Fig. 5.2.4

Transfer test performance before and after 10 acquisition trials to the platform position indicated. The swim paths of one rat trained to the NE position have been rotated 180° to aid comparisons between figures. aCSF-treated rats showed a clear bias towards the target quadrant after training (A). However, 30 mM D-AP5-treated rats never showed such a bias (B), and many were extremely thigmotaxic during the second transfer test (C).

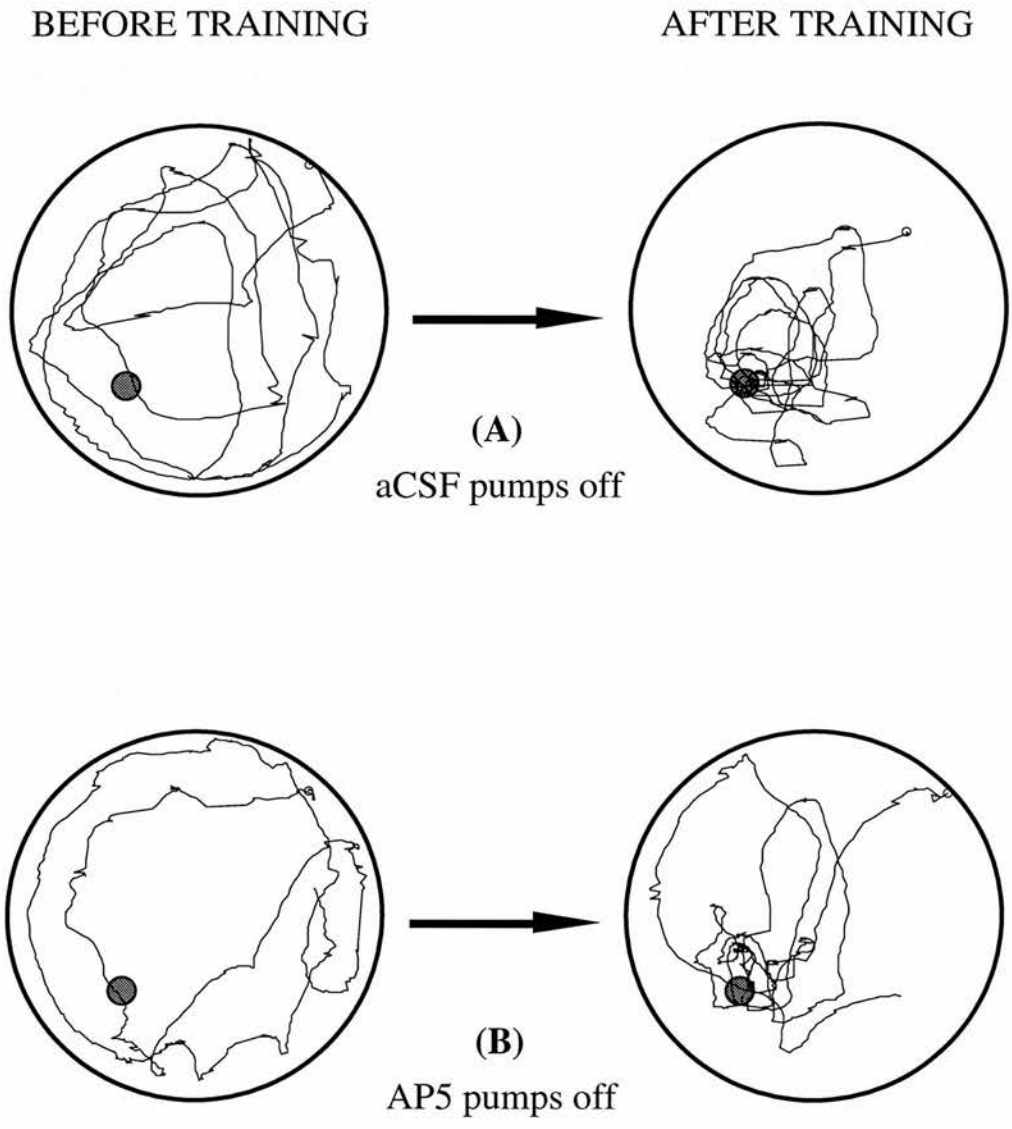


Fig. 5.2.5

Transfer test performance before and after 10 acquisition trials to the platform position indicated (pumps exhausted). Both groups showed an equivalent bias towards the training quadrant in the second transfer test.

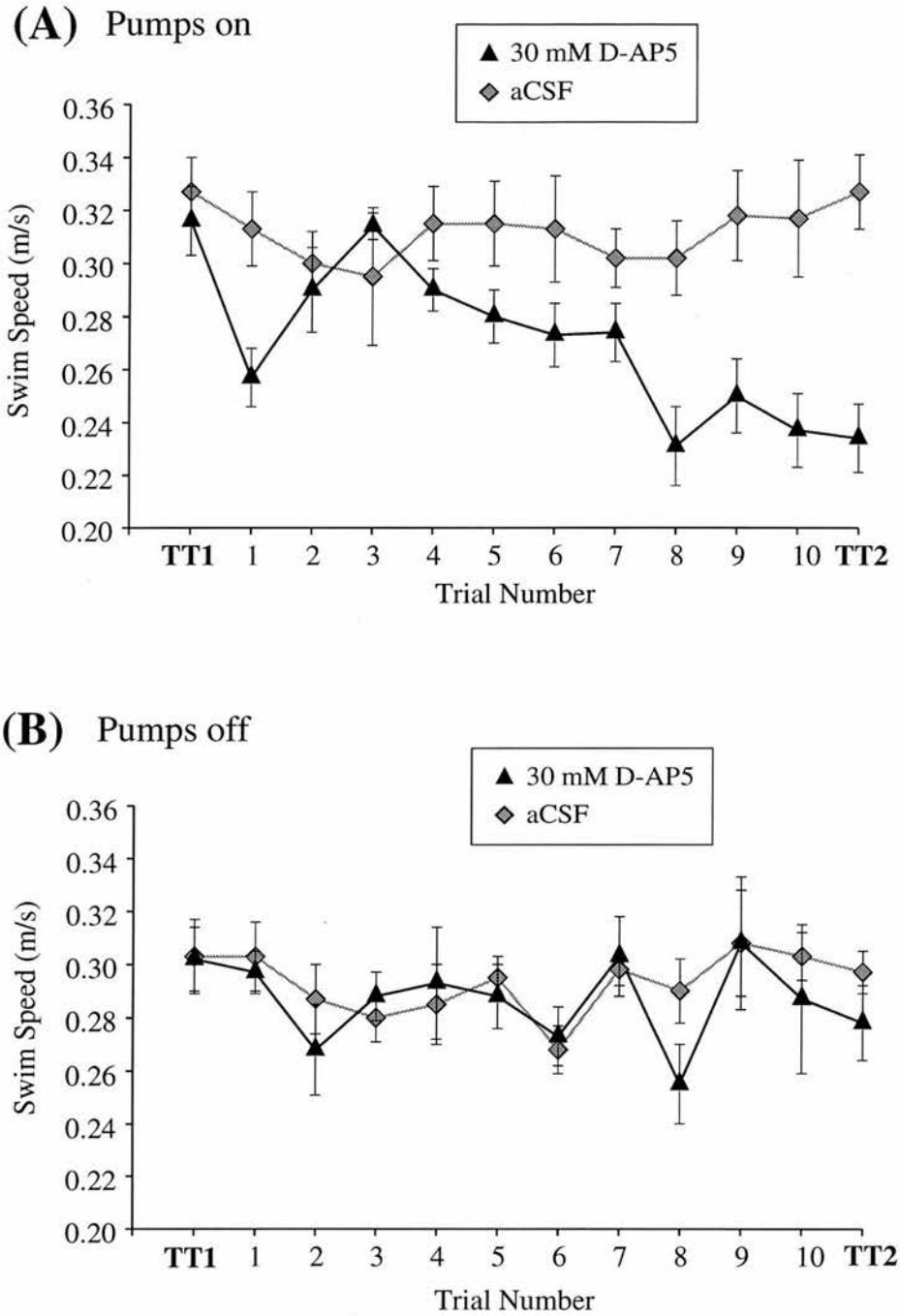
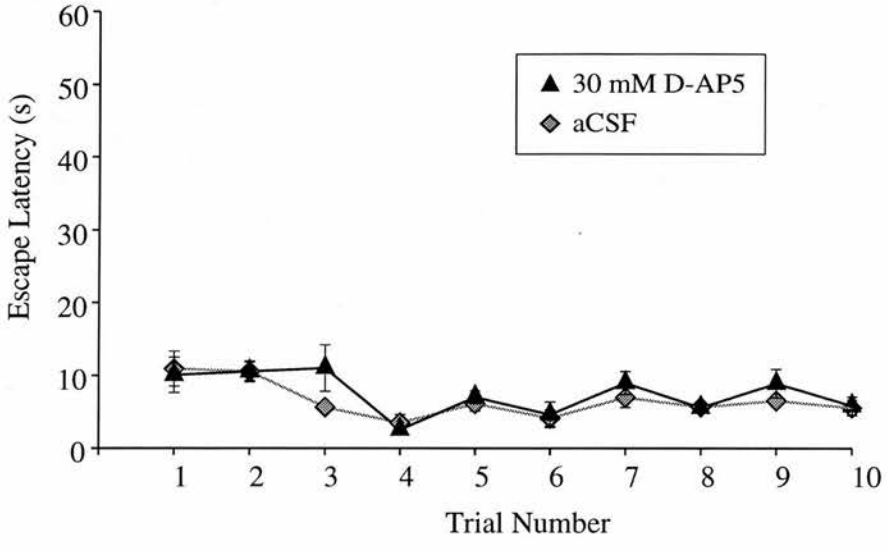


Fig. 5.2.6

(A) Mean swim speeds throughout training. Transfer tests (TT1 & TT2) are plotted together with regular trials. Rats infused with 30 mM D-AP5 swam more slowly as testing progressed. Controls, however, swam at roughly the same speed throughout. (B) No differences in swimming speed were found in rats tested after exhaustion of minipumps.

(A) Pumps on



(B) Pumps off

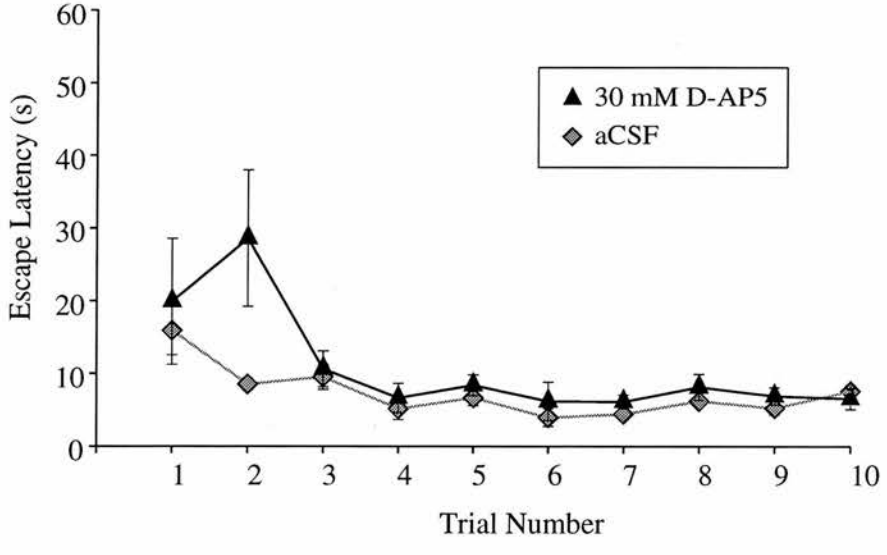
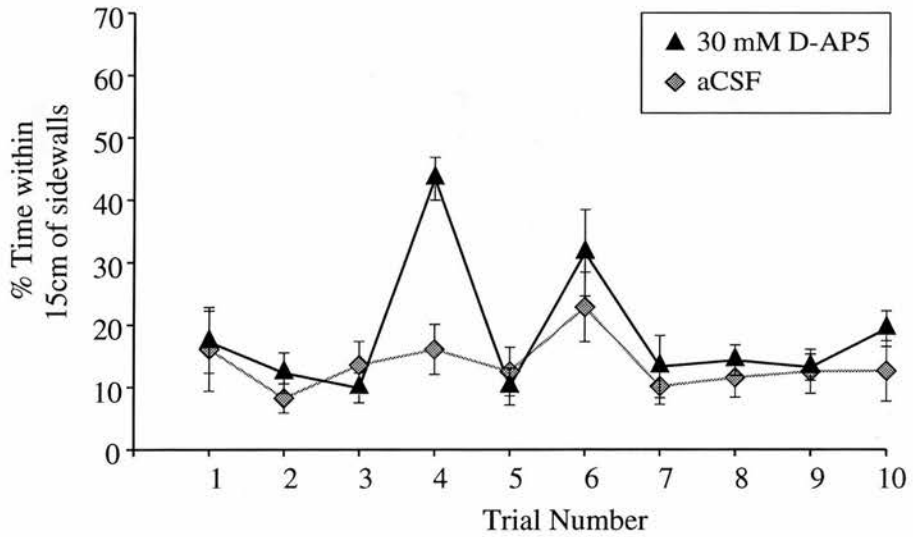


Fig. 5.2.7

(A) Minipump infusion of 30 mM D-AP5 does not impair performance of a visually-cued task in the watermaze. (B) No impairment is seen when testing is carried out after the minipumps are exhausted.

(A) Pumps on



(B) Pumps off

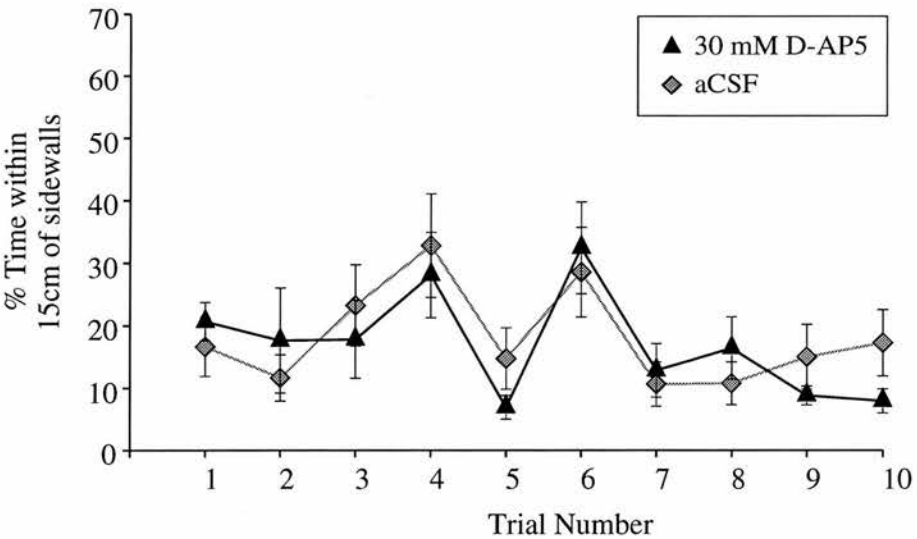
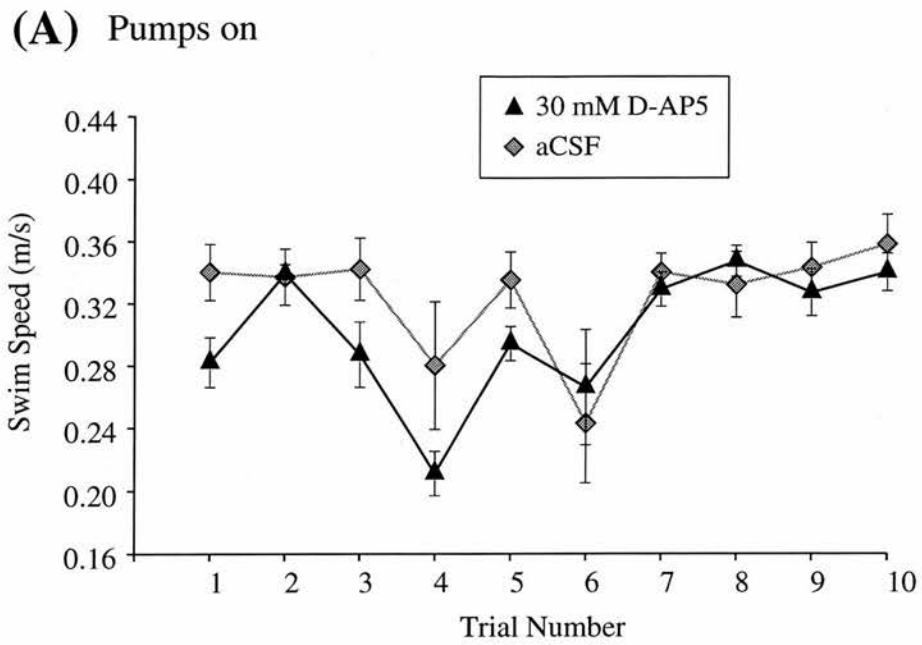


Fig. 5.2.8

(A) Percentage time spent within 15 cm of the sidewalls during visible platform testing. No group difference was seen. (B) No group difference in thigmotaxis was seen in rats tested after exhaustion of minipumps.



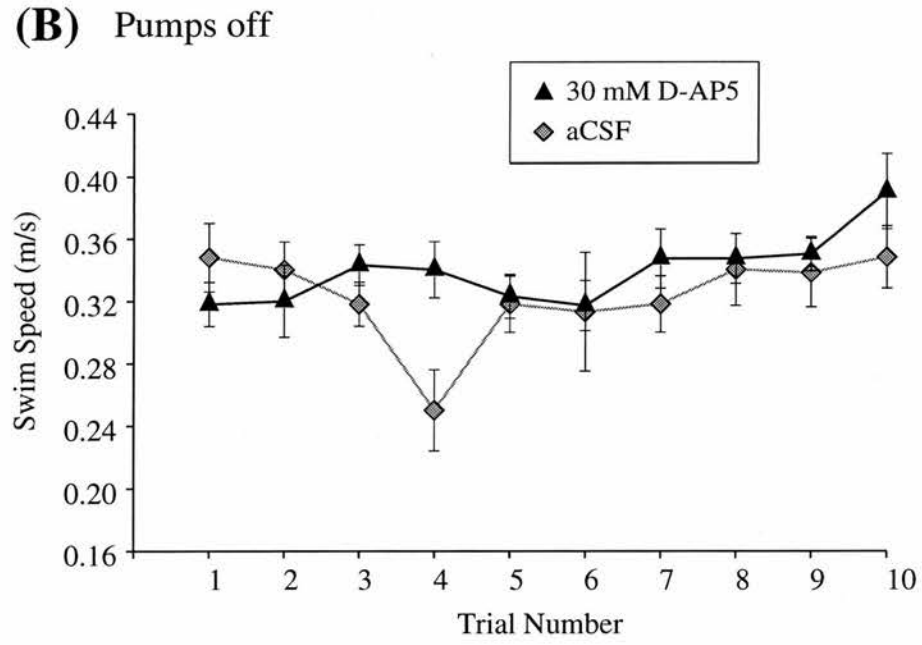


Fig. 5.2.9

(A) Mean swim speeds throughout visible platform training. No group differences in swimming speed were found. (B) No group differences in swimming speed were seen after exhaustion of minipumps.

5.3 Does chronic minipump infusion of 30 mM D-AP5 cause neuronal damage?

5.3.1 Introduction

The aim of this study was to determine whether chronic AP5 infusion causes neuronal damage. For comparison with those rats tested in experiment 5.2, separate groups of rats were infused with the non-competitive NMDA receptor antagonist, MK-801, a drug whose neurotoxic effects have been extensively studied. Neuronal damage was assessed by conventional staining, as well as immunostaining for heat shock protein 72 (HSP72) (see section 5.1.4). Histological analysis of perfused brains was carried out by J. E. Bell, F. Brannan, and J. Knox at the Department of Neuropathology, Western General Hospital.

5.3.2 Methods

5.3.2.1 Drug treatment and perfusion

As described in experiment 5.2, rats were perfused intracardially with 0.9 % saline and 10 % buffered formalin at the end of behavioural testing. Animals in the pumps on condition were sacrificed 4-6 days after the end of behavioural testing, i.e. before exhaustion of the minipumps. Animals in the pumps off condition were sacrificed approximately 7 days after the end of training. In addition to those animals that took part in experiment 5.2, an extra 4 rats were implanted with minipumps containing 30 mM D-AP5. Two of these animals were sacrificed during the period of drug infusion, at an equivalent time to those in the pumps on condition above. The remaining two were sacrificed after exhaustion of the minipumps, at an equivalent time to those in the pumps off condition.

In order to provide a positive control, 8 rats were given acute systemic injections of MK-801 (2 mg / kg). Instead of male Lister-hooded rats, female Cob Wistars were used, owing to the greater susceptibility of female rats to the neurotoxic actions of NMDA receptor antagonists (see section 5.1.4). Rats were sacrificed and perfused either 4-5 hr after MK-801 injection (n = 4), or 24 hr later (n = 4).

5.3.2.2 Histological analysis

After removal, the brains were further fixed in 10 % buffered formalin. Coronal sections were cut and processed using a VIP tissue processor, before being embedded in paraffin wax. Sections were cut at 5 μm and stained with haematoxylin and eosin (H & E) and Luxol fast blue in order to assess the

condition of neurons after drug application, and to select areas of the brain for immunohistological investigation. Coronal sections at the level of the posterior cingulate gyrus and posterior hippocampus were stained with monoclonal antibodies to HSP72 using a standard avidin-biotin complex method. Antibodies to HSP72 (Amersham, Little Chalfont, UK) were applied at a dilution of 1 in 100, and sections were pre-treated by microwaving in citric acid (pH = 6) for 15 min. The reaction product was visualized with diaminobenzidine, and the sections were counterstained with haematoxylin.

All brain sections were examined independently by two observers who were blind to the experimental history of the samples. Examination of brain sections was generally confined to sections at the level of the posterior cingulate cortex and hippocampus. However, in two of the AP5-treated brains from the pumps on condition, sections were examined throughout the entire rostrocaudal extent of the brain.

5.3.3 Results

Figure 5.3.1A shows an H & E stained section of posterior cingulate cortex taken from an AP5-treated rat in the pumps on condition. All cells are of normal appearance, and show neither vacuolation nor necrosis. In fact, no signs of neuronal damage were seen in any of the brains treated with either AP5 or aCSF, either in the pumps on or pumps off conditions. This was true throughout the entire rostrocaudal extent of the brains taken from two AP5-treated animals in the pumps on condition.

However, all rats injected with MK-801 showed evidence of neuropathology. Examination of H & E stained sections revealed prominent cytoplasmic vacuolation in layer 3 posterior cingulate neurons of those brains removed 4-5 hr after injection. An example is shown in figure 5.3.1B: the cytoplasm is shrunken and vacuolated in a large number of neurons. In brains removed 24 hr after MK-801 injection, vacuolation was no longer observed, but a number of necrotic neurons were evident, suggesting an ongoing process of vacuolation, followed either by recovery or by cell death (figure 5.3.1C).

Immunolabelling for HSP72 was never detected in any of the sections prepared from brains treated with either AP5 or aCSF. Figures 5.3.2 A & B show cingulate neurons negative for HSP72 in sections taken from an AP5 rat in the pumps on condition, and an aCSF rat in the pumps off condition, respectively). No immunolabelling for HSP72 was found in brains removed 4-5 hr after MK-801 injection, although vacuolated neurons were prominent at this stage (figure 5.3.2C). However, immunoreactivity for HSP72 was observed in a number of posterior cingulate neurons 24 hr after MK-801 infusion (figure 5.3.2D).

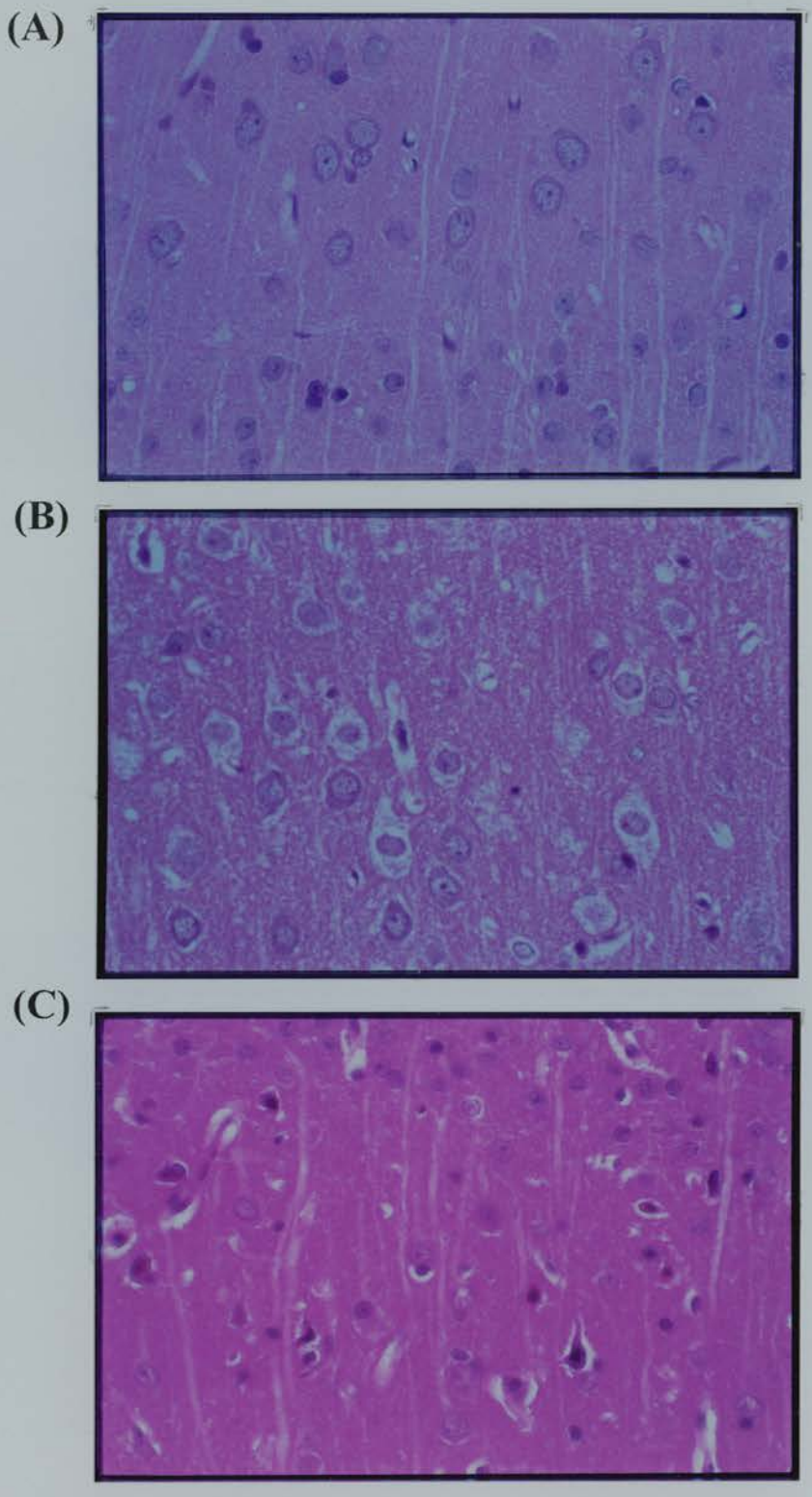


Fig. 5.3.1

H & E stained sections from the cortex of the posterior cingulate gyrus photographed at x 400. (A) D-AP5 pumps on condition: all neurons appear normal. (B) 4-5 hr after MK-801 injection: vacuolated neurons are seen in layer 3. (C) 24 hr after MK-801 infusion: necrotic neurons are visible in layer 3.

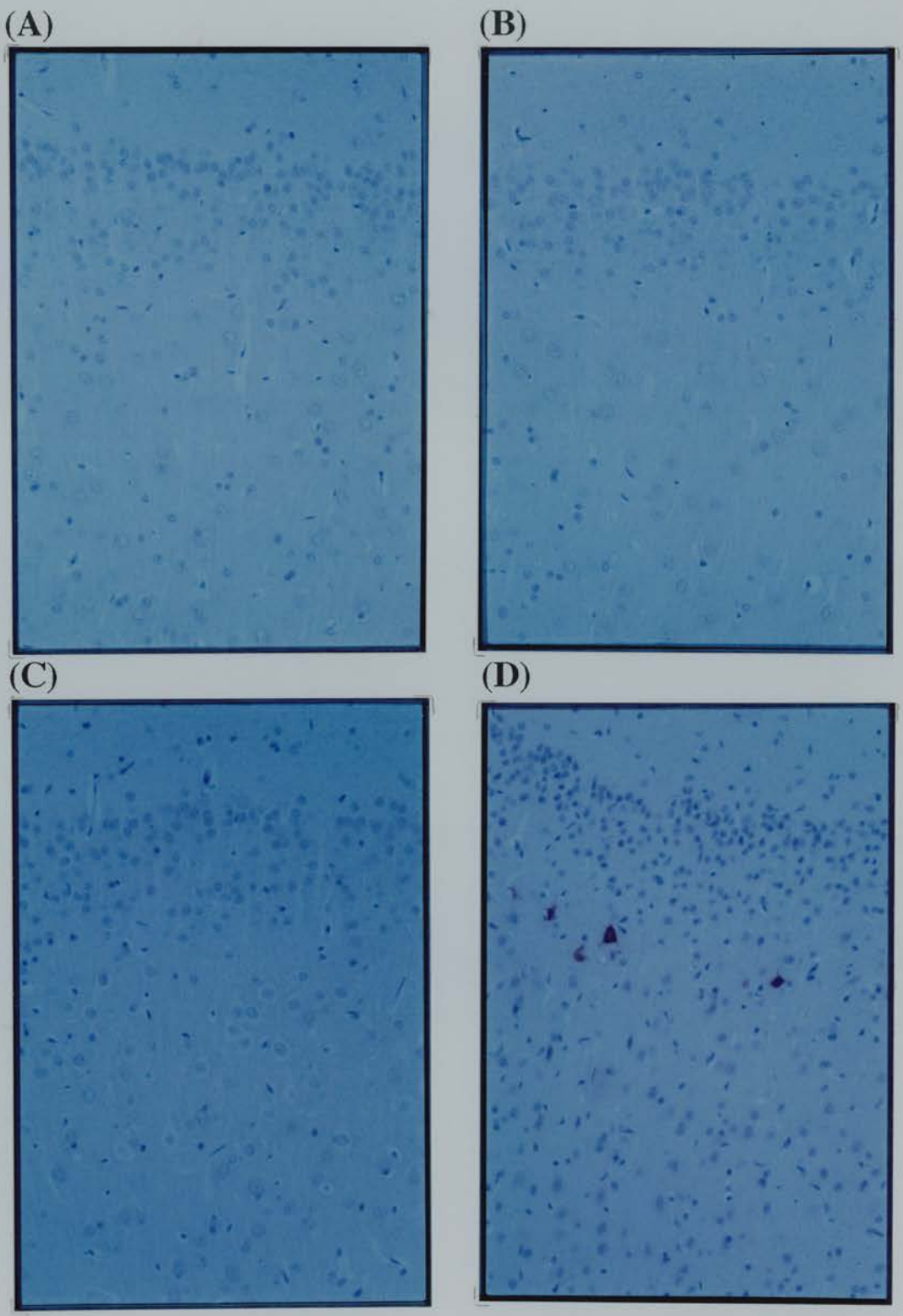


Fig. 5.3.2
Sections taken from the cortex of the posterior cingulate gyrus stained with antibodies to HSP72 (200 x magnification) (A) AP5 pumps on: HSP72 negative. (B) aCSF pumps off: HSP72 negative (C) 4-5 hr after MK-801 injection: neurons vacuolated but HSP72 negative. (D) 24 hr after MK-801 injection: occasional neurons positive for HSP72 are present in layer 3.

5.4 General discussion

5.4.1 Overview

The main finding of this study is that rats infused with 30 mM D-AP5 exhibit an impairment in watermaze reference memory performance during the period of drug infusion only, ruling out the possibility that lasting neuronal damage might account for the AP5 deficit. Nevertheless, it could still be argued that reversible neuronal injury might have contributed to the deficit in the pumps on condition. However, a detailed histological analysis of the brains of AP5-treated rats revealed no evidence of neuronal damage in either the pumps off or pumps on conditions. This result rules out the possibility that AP5 impairs learning by inducing transient neuronal damage, and confirms the absence of lasting neuropathology.

No impairment was found on a visually cued task carried out on the day following spatial training, suggesting that the behavioural impairment is limited to spatial learning. AP5-treated rats did display sensorimotor abnormalities in the course of spatial training, but a failure to learn was observed even on early trials when sensorimotor disturbances were minimal. The fact that sensorimotor abnormalities were minimal or absent at the start of training, but became progressively more severe as testing progressed, suggests that such impairments are not a necessary consequence of AP5 infusion, but may be uncovered by the use of a massed-trial training protocol, such as that used by Cain et al. (1996).

5.4.2 AP5 does not cause chronic neuronal damage

Acute intracerebral infusion of AP5 has previously been reported to cause neuronal vacuolation (Labruyere et al., 1989). However, no evidence of neuronal damage was seen in any of the AP5-treated brains in the present study. Although the chronic infusion of D-AP5 is not exactly comparable to the acute injection of MK-801, it is likely that in the pumps on condition at least, any neurotoxic effects of AP5 would be manifested by an ongoing process of vacuolation and necrosis, such as that seen in the 4-5 hr and 24 hr MK-801 groups. The absence of such pathology confirms that chronic infusion of 30 mM D-AP5 at a rate of 0.5 µl / hr is not neurotoxic.

In contrast, systemic injection of MK-801 (2 mg / kg) in female rats resulted in neuronal vacuolation in the cingulate cortex after 4-5 hr, and necrosis 24 hr later. The former result is similar to that obtained by Olney et al. (1989), and the latter confirms the finding that MK-801 induced damage may not always be reversible (Allen and Iversen, 1990; Fix et al., 1993). Consistent with previous reports (see section 5.1), 24 hr after MK-801 injection, a number of neurons within the posterior cingulate

cortex were found to be immunopositive for HSP72.

5.4.3 Sensorimotor disturbances

Rats tested in the "pumps on" condition displayed severe sensorimotor impairments, which were slight at the start of training, but grew considerably worse as testing progressed. These impairments were worse than those typically observed with the testing protocols regularly used in this laboratory, such as one trial per day (e.g. Bannerman et al., 1995), or four trials per day with a total ITI of 45 s between trials (e.g. Steele and Morris, 1999). In fact, AP5-treated rats became so fatigued towards the end of the testing session that any assessment of their learning abilities was impossible. Many rats were unable to balance on the platform at all after several trials, a situation which robs the platform of any reward value. Behavioural abnormalities included falling off the platform during a "wet-dog shake", thigmotaxic swimming around the side-walls, failure to climb onto the platform after contacting it, "swimming" over the platform, and occasionally continuing to make swimming movements even after removal from the pool. These disturbances are similar to those reported by Cain et al. (1996).

The percentage time spent within 15 cm of the sidewalls, an index of thigmotaxic swimming, was equivalent in AP5- and aCSF-treated rats at the start of testing. However, AP5-treated rats spent an increasing amount of time at the sidewalls as testing progressed (figure 5.2.3A). A striking illustration of this deterioration in search strategy is provided in figure 5.2.4C. The rat in question searched efficiently for the platform in the initial probe test with the platform absent, but after 10 trials the rat did little more than aimlessly circle the walls of the pool. Similarly, AP5-treated rats initially swam at the same speed as controls, but gradually slowed down as testing progressed, indicating increasing fatigue.

The fact that AP5-treated rats were initially unimpaired according to the above criteria, but deteriorated as testing progressed, is consistent with the suggestion that the testing protocol is responsible for the extreme levels of sensorimotor impairment obtained. It is possible that a genuine learning deficit during the initial trials leads to increasing fatigue which exacerbates the sensorimotor side effects of AP5 and hence leads to an even greater decline in performance. Hence, there is no reason to assume that sensorimotor disturbances are the cause of the "learning" impairment, it is equally likely that an initial learning impairment, coupled with the massed-trial training protocol, may have caused the severe sensorimotor disturbances.

5.4.4 Visible platform task

Despite the fact that thigmotaxis and swim speed were normal in AP5-treated rats at the start of testing, other behavioural abnormalities such as falling off the platform were evident from the start. However, falling from the platform decreases over days in protocols involving a number of testing sessions (R. G. M. Morris, R. J. Steele, S. J. Martin, and J. E. Bell, unpublished observations), another reason why a single session of 10 trials may be inappropriate.

Considering the severe sensorimotor impairments evident during hidden platform training, I fully expected AP5-treated rats to be unable to stand on the platform during visible platform training, and an impairment on this task, such as that reported by Cain et al. (1996) seemed inevitable. However, I was surprised to find that AP5-infused rats were totally unimpaired on the visually cued task. Rats which had never managed to balance satisfactorily on the hidden platform, stood on the visible platform with ease. No deficits were seen in latency to reach the platform, thigmotaxic swimming, or swimming speed. However, Cain et al. (1996) tested rats on the visible platform task on the same day as the hidden platform task, whereas I carried out the visible platform task on the following day. Hence, an improvement in performance over days, such as that described above, may explain the discrepancy between these results and those reported by Cain and colleagues. However, this assertion cannot be tested by exactly replicating the latter study, owing to Home Office regulations concerning the number of permissible watermaze trials per day.

The nature of this "overnight improvement" in performance is unclear. The new-found ability to stand on the platform may reflect a form of motor learning. It is often found that distributed training is more efficient than massed training in the acquisition of motor skills (e.g. Carron, 1969), a result which holds for a number of different procedural learning tasks. The overnight improvement in one such task (a form of visual discrimination carried out with human subjects) has been found to be dependent on REM sleep (Carni et al., 1994). If the reduction in the sensorimotor deficits associated with AP5 infusion involves procedural learning of some kind (i.e. learning to "compensate" for drug induced disturbances in performance) then the 24 hr interval between spatial and cued tasks may provide an explanation for the radical reduction in sensorimotor disturbances. Incidentally, the mere inhibition of "wet-dog shakes" is not responsible, since AP5-treated rats have a tendency to shake more than controls throughout training. In fact, the number of "wet-dog" shakes performed by drug-treated animals actually increases over days, whereas the tendency to fall off the platform very rapidly diminishes (R. G. M. Morris, R. J. Steele, S. J. Martin, and J. E. Bell, unpublished observations).

Alternatively, it is possible that the normal performance of AP5-treated rats on early trials of the visible platform task may have prevented fatigue and the associated sensorimotor deficits observed during hidden platform training. The visible platform deficit reported by Cain et al. (1996) may have resulted from fatigue effects being carried over from one task to the other, since both were conducted on the same day. Other explanations include the possibility that a reduction in the stress associated

with exposure to the watermaze may occur between days. Additionally, AP5-treated rats may, in fact, have learned about the "procedural" demands of the watermaze during hidden platform testing. Whilst this learning was not expressed at the time, perhaps owing to severe fatigue, it is possible that latent knowledge may have resulted in unimpaired performance on the visually cued task. Whatever the explanation, the absence of an impairment on the visible platform control task when carried out 24 hr after hidden platform testing provides a further illustration that sensorimotor deficits are not an inevitable consequence of AP5 infusion, but only become apparent under certain testing conditions.

5.4.5 Summary

The experiments described above do not provide conclusive evidence that NMDA receptors are involved in spatial learning *per se*, rather than other aspects of watermaze performance. Indeed, the behavioural abnormalities induced by the interaction of testing protocol and drug treatment are so severe that the possibility of a learning deficit cannot be assessed. Some behavioural disturbances, such as thigmotaxis, abnormally slow swimming, and making swimming movements after removal from the water, were not evident at the start of spatial training, but became worse as testing progressed. Other disturbances, such as falling off the platform, were evident from the start of spatial training, but declined between testing days. Hence, the severe sensorimotor disturbances observed in the current study, and that of Cain et al. (1996), cannot be used as evidence that the spatial learning deficit obtained by others, using different testing protocols, is secondary to a sensorimotor deficit. As emphasized above, severe sensorimotor disturbances result from an interaction of drug treatment and training protocol. Severe sensorimotor disturbances are not an inevitable consequence of AP5 infusion.

Rats tested after the end of a two-week period of AP5 infusion performed equally well as controls in both spatial and cued watermaze tasks, ruling out the possibility that lasting neuropathology contributes to the spatial learning deficit. In addition, a thorough histological examination of the brains of rats given chronic i.c.v. infusion of 30 mM D-AP5 failed to reveal any evidence of neuronal damage either during or after a two-week period of drug infusion. Hence, the spatial learning deficit caused by this method of AP5 infusion cannot be attributed either to permanent or to transient brain damage.

Chapter Six

The effects of the mGluR antagonist (*R*,*S*)-α-methyl-4-carboxyphenylglycine (MCPG) on spatial learning

6.1 General introduction

The results of the previous chapter support the hypothesis that NMDA receptors play a role in learning. Firstly, chronic AP5 infusion did not result in neurotoxic damage. Secondly, severe sensorimotor disturbances were not a necessary consequence of AP5 infusion, but were readily observed after multiple training trials carried out in a single day. The recent finding that intrahippocampal infusion of AP5 causes a delay-dependent deficit in a watermaze matching-to-place task (Steele and Morris, 1999) provides fairly strong evidence that a sensorimotor or motivational account cannot adequately explain the AP5 deficit.

However, the role of NMDA receptors in hippocampal functioning may extend beyond a role in LTP-like processes. Moderate doses of AP5 have been found to reduce cell excitability and to impair complex cell firing (see chapter 2.6.6). It has been suggested that NMDA receptors may be involved in the routine functioning of the hippocampal circuitry, in addition to their role in synaptic plasticity (see Bekkers and Stevens, 1990b). Hence, the finding that AP5 impairs learning does not necessarily imply the involvement of LTP-like processes in learning.

The fact that NMDA receptor antagonists cause sensorimotor disturbances, and may interfere with normal hippocampal functioning independently of their effects on synaptic plasticity, introduces inevitable confounds in all studies of the role of NMDA-receptor dependent plasticity in learning. Whilst these problems do not necessarily invalidate the conclusions of previous reports, the use of a pharmacological tool without these drawbacks would clearly be desirable. In this respect, metabotropic glutamate receptors provide an attractive alternative target for pharmacological intervention (see chapter 3.12).

Comparatively few studies of the role of mGluR antagonism in spatial learning have been carried out. Riedel et al. (1994a) found that an i.c.v. injection of MCPG (5µl, 200 mM) prior to training in a foot-shock reinforced Y-maze spatial alternation task, resulted in a deficit when rats were later tested for retention. At the time when experiment 6.2 was started, this remained the only study of the role of mGluRs in spatial learning: the role of mGluRs in watermaze performance was unknown.

In the course of this investigation, however, it was reported that a bilateral i.c.v. infusion of (*R,S*)-MCPG (5 µl / ventricle; 20 mM) could produce a deficit in the watermaze reference memory performance of rats (Richter-Levin et al., 1994). A further study of the effects of i.c.v. MCPG infusions on reference memory performance has since been published (Bordi et al., 1996), although the results of this experiment differ somewhat from those of Richter-Levin et al. (1994). Nevertheless, both studies report that MCPG causes a deficit in transfer test performance, but has little or no effect on latency to locate a hidden platform.

The aim of the present series of experiments was to characterize the impairment induced by MCPG in a spatial reference memory task, similar to the tasks subsequently carried out by Richter-Levin et al. (1994) and Bordi et al. (1996). The protocol consisted of 3 days of acquisition training followed by a transfer test and visible platform control task. MCPG was delivered either acutely via an intraventricular cannula (experiment 6.2), chronically via an intraventricular osmotic minipump (experiment 6.3) or acutely via intrahippocampal cannulae (experiment 6.4).

6.2 I.c.v. infusion of (*R,S*)-MCPG impairs the performance of rats in a watermaze spatial reference memory task.

6.2.1 Methods

6.2.1.1 Surgery

Intraventricular cannulae were implanted as described in chapter 4.4.2.

6.2.1.2 Drugs

Solutions were prepared by first dissolving the solid, crystalline form of each drug in 1M sodium hydroxide to form an equimolar solution, then diluting with 0.9% saline to achieve the desired concentration (20 and 200 mM (*R,S*)-MCPG; 100 mM (-)-MCPG). Drug solutions were stored in small aliquots at –20 °C when not in use.

6.2.1.3 Assignment to groups

Animals were randomly assigned to one of three groups, each of which received a different infusion during the acquisition phase of training. Two different concentrations of (*R,S*)-MCPG were tested: 200 mM (n = 13) and 20 mM (*R,S*)-MCPG (n = 9). A control group (n = 11) received 100 mM (-)-MCPG. The minus form of MCPG is reported to be the inactive enantiomer (Jane et al., 1993; Riedel et al. 1995a) and was given in preference to vehicle injections in order to control for possible non-specific effects of MCPG, not associated with the blockade of metabotropic glutamate receptors. A concentration of 100 mM was chosen because this matches the concentration of (-)-MCPG present in a 200 mM (*R,S*)-MCPG solution.

6.2.1.4 Behavioural testing

The watermaze reference memory task was carried out as described in chapter 4.2.2. Pre-training was carried out without prior drug infusion, but on all other days testing was carried out 30 min after the end of infusion.

Prior to the transfer test, most animals were infused with the same drug as on previous days. However, a subset of rats from the 200 mM (*R,S*)-MCPG group (n = 4) were instead given (-)-MCPG

in order to control for a possible state-dependent effect. Similarly, a subset of the (-)-MCPG control group (n = 4) were injected with 200 mM (*R,S*)-MCPG in order to test for the existence of retention or performance deficits in control subjects exposed to the active isomer for the first time.

Visible platform testing was carried out 2 days after the transfer test. Each rat received the same drug as during acquisition 30 min prior to this session.

6.2.1.5 Electrophysiology

Following behavioural testing, a number of rats from each group (200 mM (*R,S*)-MCPG: n = 5; 20 mM (*R,S*)-MCPG: n = 6; 100 mM (-)-MCPG: n = 9) were prepared for electrophysiology under urethane anaesthesia as described in chapter 4.3. Stimulation and recording were both carried out on the left-hand side of the brain, and hence contralaterally to the cannulated right ventricle.

Half an hour or more after implantation of the electrodes, a number of 700 μA test pulses were delivered to the perforant path at 20 s intervals in order to evaluate the stability of baseline responses. As soon as the fEPSP appeared to be stable, an input-output curve consisting of six pulses (whose values were averaged) at each of 11 stimulation intensity values ranging from 0-1000 μA, was recorded. From this I / O curve, the stimulation current required to elicit a population spike with an amplitude 40 % of the maximum obtained was calculated, and stimulation intensity was set at this value before a trial baseline was started; the intensity was later adjusted, if necessary, in order to maintain the desired population spike amplitude. A 25 min baseline period of 0.05 Hz stimulation was then recorded, although if at any time during this period the EPSP slope or population spike amplitude showed a systematic drift away from its initial value, the session was restarted. After 25 min, a 5 μl drug infusion was given over a period of 5 min, in the same way as described for behavioural testing. Each rat was injected with the same drug as that received during the acquisition phase of spatial learning. Tetanic stimulation (10 trains of 15 pulses at 200 Hz with 10 s between trains, and an intensity double that used for test pulses) was delivered 30 min after the completion of the injection (i.e. the moment equivalent to the start of a watermaze testing session). Field potentials were recorded for a further 2 hr after tetanization. In order to assess the efficacy of the tetanus parameters in the absence of any drug treatment, LTP was measured in an additional four rats that received no drug infusion prior to testing.

6.2.1.6 Histology

At the end of the experiment, each rat's brain was removed and preserved in 10 % formalin, before the preparation of sections for examination under the light microscope as described in chapter 4.5.

6.2.2 Results

6.2.2.1 Acquisition training

All groups showed a progressive reduction in escape latency during acquisition training (figure 6.2.1) and there was no visible sign of any sensorimotor disturbance during either swimming or climbing onto the platform. An ANOVA of escape latencies over the 3 days of spatial training revealed a highly significant overall improvement across trials [$F(17,510) = 6.26$; $p < 0.0001$] but no significant group difference [$F(2,30) = 1.86$; $p > 0.1$]. Inspection of figure 6.2.1 suggests that 200 mM MCPG-treated rats took slightly longer to reach the platform on the very first training trial. A separate ANOVA of trial 1 performance only, revealed a non-significant trend towards a group difference on this trial [$F(2,30) = 2.58$; $p < 0.1$].

6.2.2.2 Swim speeds

An ANOVA of swim speeds over the three days of acquisition training revealed a significant effect of trial, since rats tended to slow down within each testing session, particularly on the first day [figure 6.2.2; $F(17,510) = 2.11$; $p < 0.01$]. However, no significant overall group difference was seen [$F(2,30) = 1.30$; $p > 0.2$].

6.2.2.3 Thigmotaxic swimming

An ANOVA of the percentage time spent within 15 cm of the side-walls throughout training revealed a significant decrease in this index of thigmotaxis over successive trials [figure 6.2.3; $F(17,510) = 4.27$; $p < 0.0001$]. However, there was no group difference in thigmotaxic swimming [$F < 1$].

6.2.2.4 Transfer test and state dependent controls

An ANOVA of time spent in different quadrants of the pool during the transfer test revealed a significant overall bias towards the quadrant in which the platform was situated during acquisition training (the "training quadrant") [figure 6.2.4A; $F(3,90) = 27.2$; $p < 0.0001$] as well as a significant group by quadrant interaction [$F(6,90) = 3.68$; $p < 0.01$]. Analysis of simple effects revealed that both (-)-MCPG and 20 mM (*R,S*)-MCPG groups showed a significant bias towards the training quadrant [(-)-MCPG: $F(3,90) = 20.3$; $p < 0.001$; 20 mM (*R,S*)-MCPG: $F(3,90) = 11.2$; $p < 0.001$]. However, no such bias was evident in the 200 mM (*R,S*)-MCPG group, who performed at chance [$F(3,90) = 1.72$;

$p > 0.1$]. A separate ANOVA of percentage time spent in the training quadrant only, revealed a significant overall group difference [$F(2,30) = 5.44$; $p < 0.01$]. *Post hoc* Newman-Keuls pairwise comparisons indicated that the 200 mM (*R,S*)-MCPG group performed significantly worse than both the (-)-MCPG group [$p < 0.01$] and the 20mM (*R,S*)-MCPG group [$p < 0.05$]. No significant difference was found between (-)-MCPG and 20 mM (*R,S*)-MCPG groups. Examples of the swim paths taken by rats in each group are shown in figure 6.2.5.

Breakdown of the data into state dependency groups revealed that the subset of 200 mM (*R,S*)-MCPG rats injected with (-)-MCPG during the transfer test showed no significant difference, in terms of time spent in the training quadrant, to the majority injected with 200 mM (*R,S*)-MCPG [figure 6.2.4B; *post hoc* Newman-Keuls pairwise comparison; NS]. The group of control rats injected with 200 mM (*R,S*)-MCPG during the transfer test also showed no significant difference to those injected with (-)-MCPG [*post hoc* Newman-Keuls pairwise comparison; NS]. Sample swim paths are shown in figure 6.2.6.

Richter-Levin et al. (1994; p856) stated that "most MCPG-treated rats swam initially towards the location where the platform had been in the same way as did control or vehicle-injected rats. Subsequently, the MCPG-treated animals spent more time away from this location than control or vehicle-injected animals, leading to the significant differences observed in the quadrant analysis." In order to test this possibility, a measurement of latency to reach the previous position of the platform was taken (figure 6.2.7). However, the increase in mean latency shown by the MCPG high group was not found not to be significant: An ANOVA showed no overall differences between the three groups [$F(2,30) = 2.42$; $p > 0.1$].

To address the issue in more detail, the amount of time spent in the training quadrant during the 60 s transfer test was re-analysed by division into four 15 s time windows (figure 6.2.8). Owing to the small size of the state dependent control groups (n = 4), and the absence of state dependent effects in the overall analysis, data from sub-groups were pooled for the following analysis. An ANOVA comparing time spent in the training quadrant only at each time interval revealed, as expected, a significant effect of group [$F(2,30) = 5.42$; $p < 0.01$] as well as an effect of time interval [$F(3,90) = 2.82$; $p < 0.05$], suggesting that an overall change in spatial bias did occur over the course of the transfer test. In fact, overall performance was marginally worse between 45-60 s than between 30-45 s, probably due to extinction [*post hoc* Newman-Keuls pairwise comparisons; $p < 0.05$]. However, no significant group by interval interaction was found [$F < 1$]. This last result does not support the idea that MCPG-treated rats searched initially in the correct location, then subsequently spent more time in other areas of the pool. Indeed, an ANOVA of performance in the first 15 s of the transfer test still revealed a highly significant difference between the 200 mM MCPG group and (-)-MCPG-treated controls [Newman-Keuls *post hoc* pairwise comparison; $p < 0.01$].

6.2.2.5 Visually cued task

On the day of visible platform testing, the cannula became detached from the skull of one rat in the 200 mM (*R,S*)-MCPG group during infusion; a second rat in the (-)-MCPG group became ill and was dropped from the study. Hence, data from these animals was not available for the following analysis. An ANOVA of the escape latencies of the remaining animals (n = 31) revealed a significant overall improvement across trials [figure 6.2.9A; $F(3,84) = 3.02$; $p < 0.05$], but also a significant effect of group [$F(2,28) = 3.46$; $p < 0.05$]. However, the group x trial interaction did not reach significance [$F(6,84) = 1.23$; $p > 0.2$]. Individual *post hoc* pairwise comparisons (Newman-Keuls) revealed a significant difference between the 200 mM (*R,S*)-MCPG and (-)-MCPG groups [$p < 0.05$]. The 20 mM (*R,S*)-MCPG group did not differ significantly from either of the other two groups. However, an analysis of simple effects revealed that all groups had reached an equivalent level of performance by trial 4.

It was noticed during testing that whilst control animals almost always swam directly to the platform, 200 mM (*R,S*)-MCPG-treated rats often appeared to orient themselves towards the visible platform and maybe even begin an approach, before turning away, searching briefly elsewhere, then finally heading for the platform again. An example of the path taken by an (*R,S*)-MCPG-treated rat behaving in this way is shown in figure 6.2.9B. This result would tend to indicate a possible attentional or motivational deficit in MCPG-treated animals, rather than a simple visual or motor impairment.

6.2.2.6 EPSP Slope Potentiation

The EPSP slope potentiation data are shown in figure 6.2.10A. All values were normalized to the mean EPSP slope over the 10 min immediately prior to tetanization. PTP was defined as the mean slope value over the 4 min immediately following tetanization. LTP was calculated as the mean EPSP slope value measured over one of two 10-min periods: 50-60 min post-tetanus, and 110-120 min post-tetanus. Separate ANOVAs of the normalized EPSP slope at these different time intervals after tetanization failed to reveal a significant group difference at any point (table 6.2.1; 200 mM (*R,S*)-MCPG: n = 5; 20 mM (*R,S*)-MCPG: n = 6; 100 mM (-)-MCPG: n = 9). However, no group exhibited EPSP slope LTP lasting for 2 hr, hence the absence of a group difference cannot be taken as evidence against a role of mGluRs in LTP. In order to rule out the possibility that the failure to induce LTP was caused by an undocumented action of the minus form of MCPG, LTP was measured in 4 rats, selected randomly from the remaining subjects of the behavioural study, in which no infusion was given prior to tetanization. Figure 6.2.10B shows EPSP slope potentiation data from the (-)-MCPG group alongside data from the non-infused controls. No difference in potentiation was observed at any time point, and neither group showed lasting LTP 2 hr after tetanization (EPSP slope 110-120 min post tetanus: non-injected group = 103.9 ± 3.2 %; (-)-MCPG group = 101.8 ± 3.4 %).

Table 6.2.1 EPSP Slope Potentiation

	% PTP 0-4 min post–tetanus	% LTP 50-60 min post-tetanus	% LTP 110-120 min post-tetanus
100 mM (-)-MCPG	130.6 ± 4.8 %	110.2 ± 2.9 %	101.8 ± 3.4 %
20 mM (*R,S*)-MCPG	130.2 ± 4.2 %	106.6 ± 1.3 %	101.0 ± 2.4 %
200 mM (*R,S*)-MCPG	126.3 ± 2.3 %	105.2 ± 2.7 %	96.8 ± 3.9 %

ANOVA result	$F < 1$	$F < 1$	$F < 1$

6.2.2.7 Population spike potentiation

A similar analysis of population spike data revealed no significant group differences at any point after tetanization, including 110-120 min; the apparently reduced population spike LTP at this point did not reach significance (Fig. 6.2.11; Table 6.2.2; 200 mM (*R,S*)-MCPG: n = 5; 20 mM (*R,S*)-MCPG: n = 5; 100 mM (-)-MCPG: n = 8; the reduced numbers result from the omission of data from two rats owing to the fact that values had fallen very nearly to zero prior to tetanization). However, very little lasting LTP was induced in either the 100 mM (-)-MCPG or 20 mM (*R,S*)-MCPG groups, making it impossible to draw any strong conclusions from these results. In two animals tested, the population spike amplitude prior to tetanization was very close to zero, one from the (-)-MCPG group and one from the 20 mM (*R,S*)-MCPG group; these cases were excluded from the above analysis of population spike LTP.

Table 6.2.2 Population Spike Potentiation

	% PTP 0-4 min post–tetanus	% LTP 50-60 min post-tetanus	% LTP 110-120 min post-tetanus
100 mM (-)-MCPG	257.3 ± 38.0	165.1 ± 24.7	140.8 ± 26.6
20 mM (*R,S*)-MCPG	326.8 ± 43.1	193.7 ± 20.7	138.4 ± 8.5
200 mM (*R,S*)-MCPG	211.8 ± 25.2	135.5 ± 21.1	78.4 ± 11.0

ANOVA result	$F(2,15) = 2.20$; $p > 0.1$	$F(2,15) = 1.41$; $p > 0.2$	$F(2,15) = 2.47$; $p > 0.1$

6.2.2.8 Baseline parameters

Table 6.2.3 shows initial EPSP slope and population spike amplitude values, averaged over the 10 min prior to tetanization. No significant group differences were found in either measure. Baseline stimulation intensities likewise did not differ across groups. However, for a discussion of factors which were found to influence the magnitude of LTP in this study, see chapter 9.2.

Table 6.2.3 Baseline stimulation parameters

	Test pulse stimulation intensity (μA)	**Pre-tetanus EPSP slope (mV/ms)**	**Pre-tetanus population spike amplitude (mV)**
100 mM (-)-MCPG	346.0 ± 32.2	4.36 ± 0.42	2.19 ± 0.54
20 mM (*R,S*)-MCPG	441.7 ± 57.4	5.16 ± 0.43	2.61 ± 0.68
200 mM (*R,S*)-MCPG	336.0 ± 36.7	3.93 ± 0.46	2.21 ± 0.45
ANOVA result	$F(2,17) = 1.82$; $p > 0.1$	$F(2,17) = 1.87$; $p > 0.1$	$F < 1$

6.2.2.9 Histology

Coronal sectioning of the preserved brains revealed that cannulae were correctly sited in the right lateral ventricle in all cases. Slight evidence of infection at the base of the cannula was present in several of the brains, but was often particularly severe in rats that became ill and were dropped from the experiment. Infection was characterized by enlargement of the right lateral ventricle and infiltration of white blood cells. The use of isotonic saline as the vehicle for drug delivery was not ideal, since it has been reported to act as an irritant (see Urquart et al., 1984). Infection may have been caused by bacterial contamination of the injection needles or stylets. A subjective assessment was made of the severity of infection in rats that remained healthy throughout testing: all groups were equally affected, and the degree of infection was not correlated with watermaze performance (data not shown). Hence, the presence of infections around the cannula site cannot account for the effects of (*R,S*)-MCPG on spatial learning.

6.2.3 Discussion

These experiments reveal that i.c.v. infusion of MCPG disrupts the performance of rats in a spatial learning task. Although performance during acquisition was not significantly affected (figure 6.2.1), the 200 mM (*R,S*)-MCPG group showed a clear deficit when tested for retention in a subsequent transfer test (figure 6.2.4A). This deficit remained similar whether or not the active isomer was re-administered prior to testing, suggesting the absence of state-dependent effects (figure 6.2.4B). Furthermore, control animals injected for the first time with 200 mM (*R,S*)-MCPG prior to the transfer test, showed no impairment relative to controls which again received the minus form. This suggests that MCPG neither causes an impairment in the recall of previously learned spatial information, nor does it induce a detectable sensorimotor or motivational deficit in control animals under these circumstances.

These results are broadly consistent with those reported by Richter-Levin et al. (1994), who found only a marginal effect of (*R,S*)-MCPG on escape latency during acquisition, but a severe impairment in transfer test performance. As in the present study, (*R,S*)-MCPG infusion during acquisition training resulted in a transfer test deficit 24 hr later, regardless of whether or not rats were injected with a further dose beforehand.

However, the effective dose found in the present study was 5 µl of 200 mM (*R,S*)-MCPG, whereas Richter-Levin et al. report a similar behavioural impairment with a bilateral injection of 20 mM (*R,S*)-MCPG, 5 µl per ventricle, a five-fold lower concentration. However, the unimpaired 20 mM (*R,S*)-MCPG group in the current study received only half this dose, i.e. a 5µl unilateral infusion. Little is known about the mobility and binding properties of MCPG within the brain, but it is possible that differences between unilateral and bilateral infusion may also contribute to this apparent discrepancy. Unilateral infusion of 20 mM MCPG may cause a blockade of mGluRs ipsilateral to the infusion site only, without significantly affecting the contralateral forebrain.

Richter-Levin et al. (1994) made an anecdotal claim that MCPG-treated rats "swam initially towards the location where the platform had been in the same way as did control or vehicle-injected rats." Consistent with this possibility, 200 mM (*R,S*)-MCPG-treated rats in the present study were certainly able to learn something about the platform position, as the decrease in escape latency during spatial acquisition demonstrates. Furthermore, although the 200 mM (*R,S*)-MCPG group took, on average, roughly twice as long to reach the original platform position as both other groups, this difference was found not to be significant (figure 6.2.7).

However, escape latency is a more variable measure of performance than the time spent in each quadrant during a transfer test. A breakdown of the 60 s transfer test data into four 15 s time windows failed to reveal a pattern of good performance early on, followed by more rapid extinction in the 200 mM (*R,S*)-MCPG group (figure 6.2.8). In fact, rats treated with 200 mM (*R,S*)-MCPG performed best

in the interval between 30-45 s after the start of the transfer test. (Note that the transfer test carried out by Richter-Levin et al. lasted only 30 s.) Hence, the MCPG-induced deficit in the present study cannot be accounted for by differences in the rate of extinction, or persistence in searching.

The findings of a later study by Bordi et al. (1996) differ more substantially from those of the present study. Intraventricular infusion of 5µl 20 mM (+)-MCPG (20.8 µg) was found to cause a modest impairment in escape latencies during watermaze acquisition training, relative to vehicle-injected controls, as was reported by Richter-Levin et al. (1994). However, in contrast to the results of the latter study, a transfer test deficit was only seen when a further infusion of (+)-MCPG was given prior to testing. Rats unilaterally infused with (+)-MCPG throughout acquisition training, but infused with vehicle prior to the transfer test performed as well as controls infused with vehicle throughout.

In order to explain this discrepancy, Bordi et al. (1996) argue that, "Because MCPG-treated animals initially swim towards the correct quadrant, but subsequently spend more time away from this location (Richter-Levin et al., 1994), the difference between the two studies can be explained by the fact that we allowed the animals less time (20 vs. 30 sec) to search for the platform in the probe trial." However, the argument is far from compelling. It must be supposed that MCPG given only during acquisition simply decreases the persistence of searching in a drug-free transfer test; hence the absence of a deficit with a 20 s transfer test (Bordi et al., 1996), but the presence of a deficit with a 30 s transfer test (Richter-Levin et al., 1994).

This issue could potentially be settled by analysis of the change in transfer test performance over time in the present study for the group given 200 mM (*R,S*)-MCPG during acquisition, but 100 mM (-)-MCPG before the transfer test. Unfortunately, the small state-dependent group sizes used, and the increase in variability associated with division of the transfer test data into distinct time windows, mean that this analysis is not feasible.

However, Bordi et al., (1996) also found that rats infused with vehicle during acquisition training, but infused with (+)-MCPG prior to the transfer test, were impaired relative to rats infused with saline throughout. This impairment was as severe as that seen in the group given (+)-MCPG throughout. It was suggested that MCPG might impair memory retention, rather than acquisition. However, it is difficult to reconcile such an interpretation with the results of the present study, in which MCPG given during acquisition impaired transfer test performance regardless of whether the drug was re-administered beforehand. The pattern of results reported by Bordi et al. (1996) is, in fact, equally consistent with the possibility of an MCPG-induced performance deficit, unrelated to learning.

One methodological factor that may be relevant is the time interval between drug infusion and behavioural testing. Bordi et al. (1996) gave infusions 5 min prior to testing, compared to the 20 min interval used by Richter-Levin et al. (1994), or the 30 min interval used in the present study. Nevertheless, it is not clear how this factor could explain the differences between studies, and the

issues raised above remain unresolved (see section 6.1).

6.2.3.1 Is the impairment mnemonic?

Rats injected with 200 mM (*R,S*)-MCPG showed no obvious sensorimotor deficits, and an analysis of swim speeds and thigmotaxic swimming revealed no significant differences between groups (figures 6.2.2 and 6.2.3). Furthermore, the transfer test performance of control rats was not impaired by the prior injection of 200 mM (*R,S*)-MCPG in place of the minus form, and (*R,S*)-MCPG-injected rats showed no signs of the ataxia often associated with D-AP5 treatment. However, the 200 mM (*R,S*)-MCPG-treated rats did show a trend towards longer escape latencies on trial one of acquisition (figure 6.2.1), when nothing is known about the platform position, and a final testing session in which rats were required to locate a randomly-positioned prominent visible platform, with extra-maze cues obscured, revealed a significant overall impairment in the 200 mM (*R,S*)-MCPG group (figure 6.2.9), although (*R,S*)-MCPG-treated rats did reach the same level of performance as controls by trial 4. Nevertheless, considering the apparent ease of the task – control rats reach the platform within seconds – this is a rather surprising result. No other reports of such a deficit exist. Richter-Levin et al. (1994) did not carry out a visible platform task, but Bordi et al. (1996) found no effect of 20 mM (+)-MCPG on performance in a visually-cued watermaze task. Nevertheless, it may be significant that the effective dose of MCPG used by Bordi et al. (1996) was five-fold lower than the 200 mM (*R,S*)-MCPG used in the present study.

This impairment may represent a visual or sensorimotor deficit. MCPG has been reported to inhibit the transmission of nocioceptive information in the ventrobasal thalamus (Salt and Eaton, 1994), and impairs contrast adaptation in cat striate cortical cells (McLean and Palmer, 1996). It is also possible that MCPG causes attentional or motivational changes. For instance, the group I mGluR antagonist (*S*)-4C3HPG has anxiolytic properties (Chojnacka-Wojcik et al., 1997). It is not known whether MCPG shares these anxiolytic actions, but a reduction in the anxiety induced by exposure to the watermaze might reduce the motivation to locate the platform rapidly. Hence, at this stage, the possibility of non-mnemonic contributions to the poor performance of 200 mM (*R,S*)-MCPG treated rats cannot be ruled out.

6.2.3.2 Does MCPG block LTP?

The electrophysiological results presented here are inconclusive, partly because little LTP was seen in the control group. The drug concentrations and tetanus parameters used were originally chosen to match those of a previous electrophysiological study by Riedel et al. (1994a), in which it was reported that the 200 mM concentration caused a complete block of both STP and LTP, whereas a 20

mM concentration spared a short-term potentiation which fell to baseline within 1-2 hours. This study was carried out in freely moving animals, rather than under terminal urethane anaesthesia, and pulse width, rather than stimulation current was doubled during tetanization. The doubling of stimulus intensity in the present study, rather than stimulus duration, may have contributed to the poor LTP obtained.

The absence of differences in LTP between drug groups may simply have reflected a general failure to activate mGluRs. Immunocytochemical studies have revealed that group I mGluRs are perisynaptically located, outside of the postsynaptic membrane specialization (Luján et al., 1996), whilst an extrasynaptic location has been reported for group II mGluRs (Shigemoto et al., 1997). A weak tetanus may not induce sufficient glutamate release to activate mGluRs in the periphery of (or outside) the synaptic cleft, and hence a blockade of these receptors with MCPG will have no effect under these circumstances.

However, Riedel et al. (1995a) reported a block of both STP and LTP with 200 mM (*R,S*)-MCPG. The failure of this dose of MCPG to block STP in this study is difficult to reconcile with the fact that tetanization was, if anything, weaker, judging by the lack of lasting LTP. Unidentified factors, such as differences in electrophysiological equipment, or the strain of rat used, may also have contributed to the differences between the two studies. For instance, Riedel et al. (1995) used Wistar rats throughout. This strain has recently been found to express LTD more readily than Lister-hooded rats (Manahan-Vaughan and Reymann, 1997b), and it is possible that strain differences may also be found in the induction of LTP.

These problems were overcome in a subsequent series of experiments by the use of a stronger tetanus that reliably induces lasting LTP in controls (see chapter 8).

6.2.3.3 Summary

The results of the present study confirm that i.c.v. administration of (*R,S*)-MCPG impairs performance in a spatial reference memory task in the watermaze. However, the slight deficit on the visible platform control task means that it cannot conclusively be argued that the impairment in "spatial learning" is genuinely mnemonic in nature.

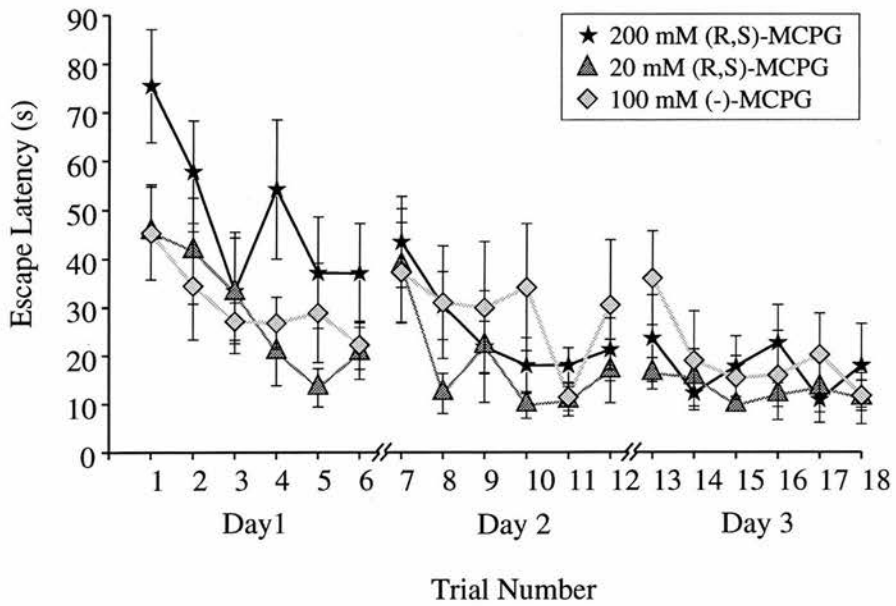


Fig. 6.2.1

Mean latency to find the hidden escape platform during acquisition of a reference memory task in the watermaze. Rats infused with 200 mM (R,S)-MCPG prior to each daily testing session were slightly impaired on the first day of testing only.

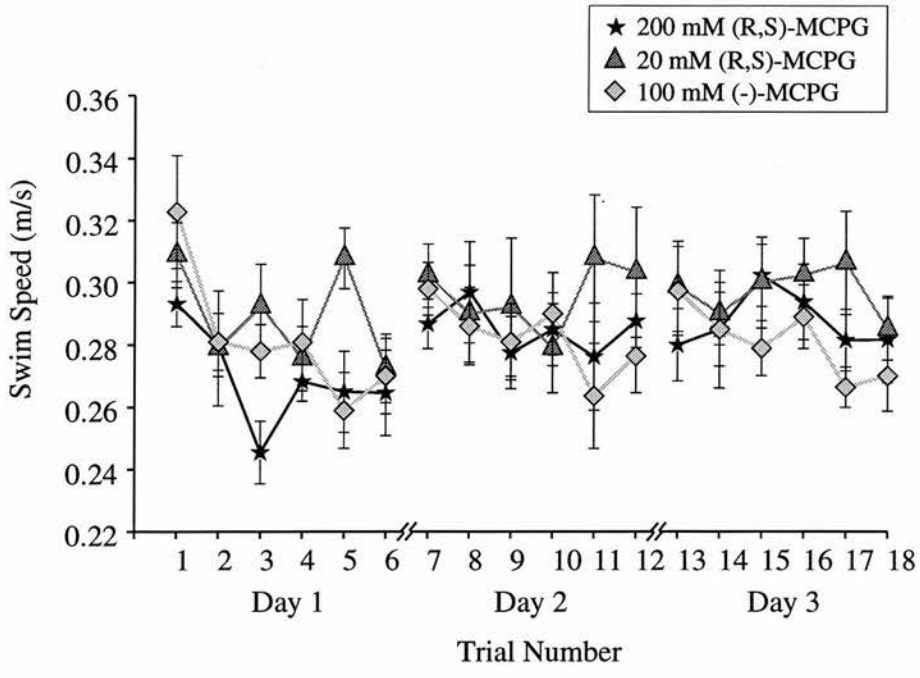


Fig. 6.2.2

Mean swim speeds during acquisition of a reference memory task in the watermaze. Swim speeds did not differ across groups.

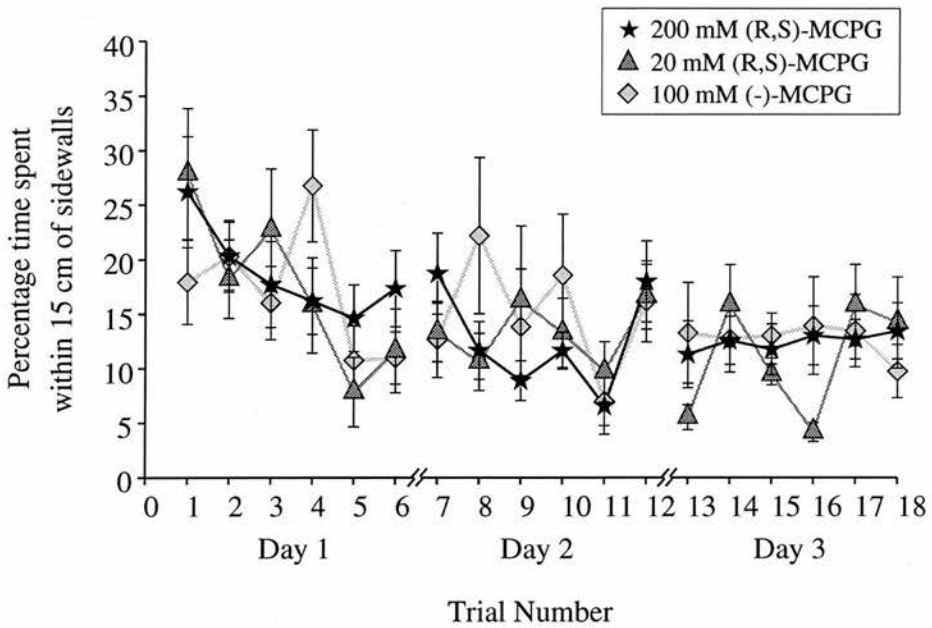


Fig. 6.2.3

Percentage time within 15 cm of the side-walls during acquisition of a spatial reference memory task. All groups were equally thigmotaxic according to this measure.

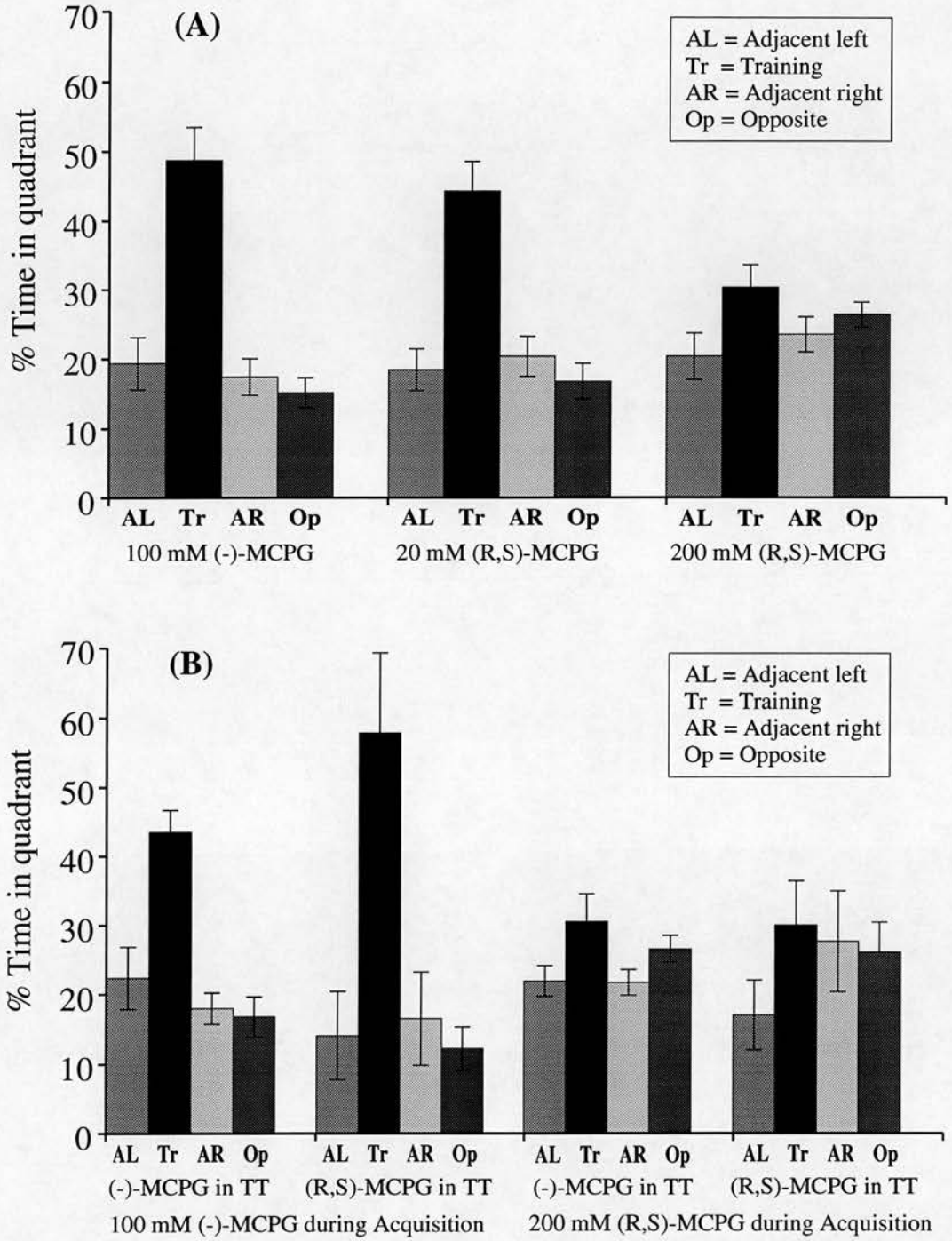


Fig. 6.2.4

(A) Mean percentage time spent within each quadrant of the pool during the transfer test. Labels below the x-axis indicate the drug infused during acquisition. (B) Transfer test performance of rats infused with 200 mM (R,S)-MCPG and 100 mM (-)-MCPG during acquisition, divided into state dependent control sub-groups according to the drug delivered prior to the transfer test (TT). Performance was determined solely by the drug delivered during acquisition training.

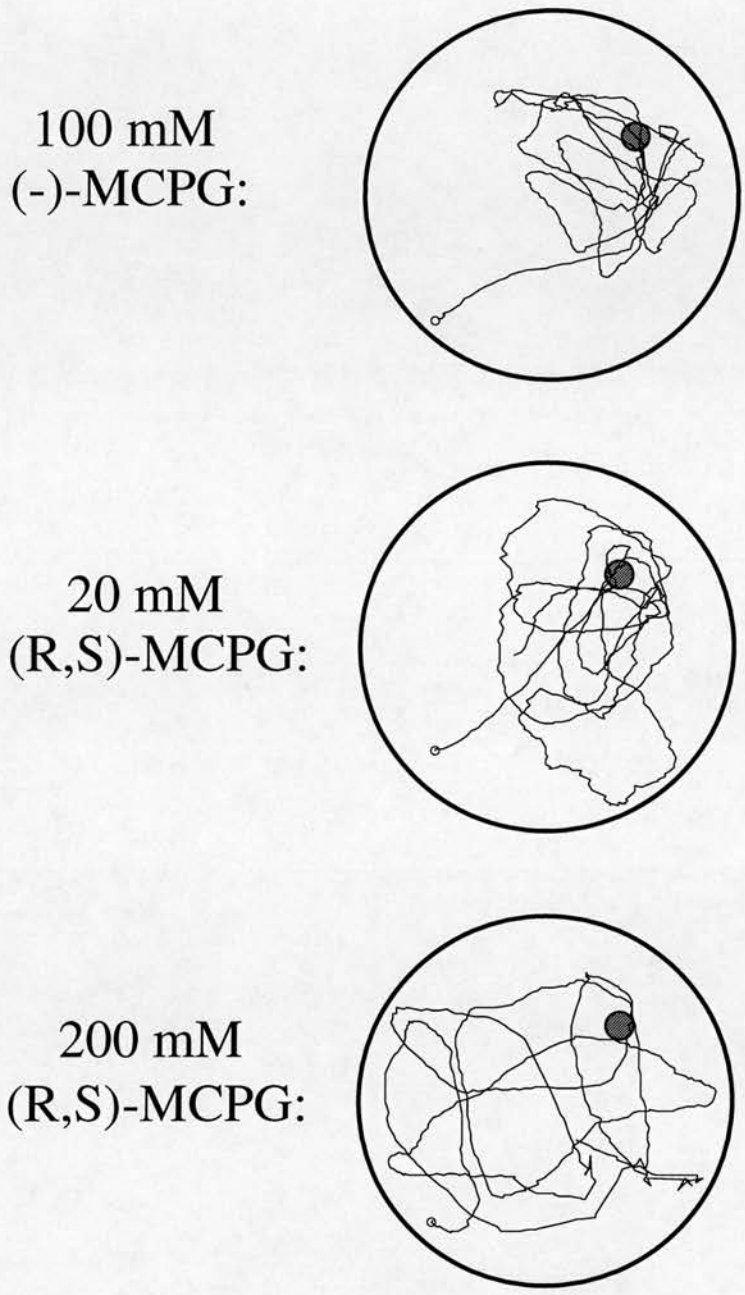
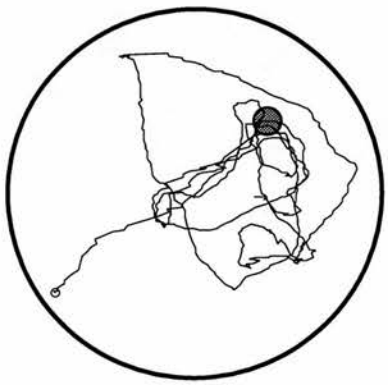


Fig. 6.2.5

Representative swim paths during the transfer test. Rats infused with 200 mM (R,S)-MCPG failed to show a significant bias towards the training quadrant. In all examples shown, rats were infused with the same drug prior to the transfer test as during acquisition training. The swim path of one rat trained to the SE position has been rotated 180° in order to aid comparisons between rats.

100 mM (-)-MCPG
during acquisition

200 mM (R,S)-MCPG
during transfer test



200 mM (R,S)-MCPG
during acquisition

100 mM (-)-MCPG
during transfer test

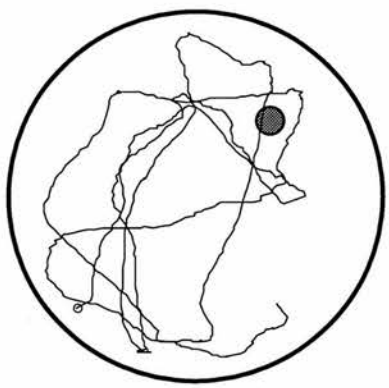


Fig. 6.2.6

Examples of the transfer test performance of state dependent control animals. Performance was determined by the drug given during acquisition training, not the drug infused prior to retention testing. The swim path of one rat has been rotated 180° in order to aid comparison of the two figures.

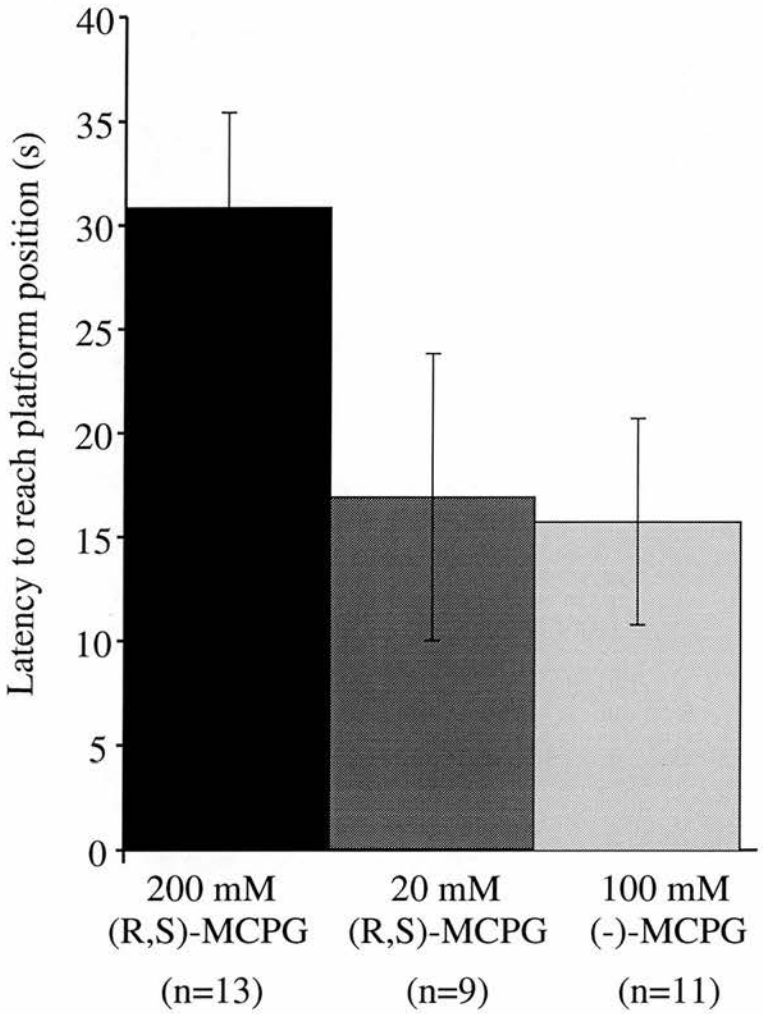


Fig. 6.2.7

Mean latencies to reach the original position of the now absent platform during the transfer test. The trend across groups does not reach significance.

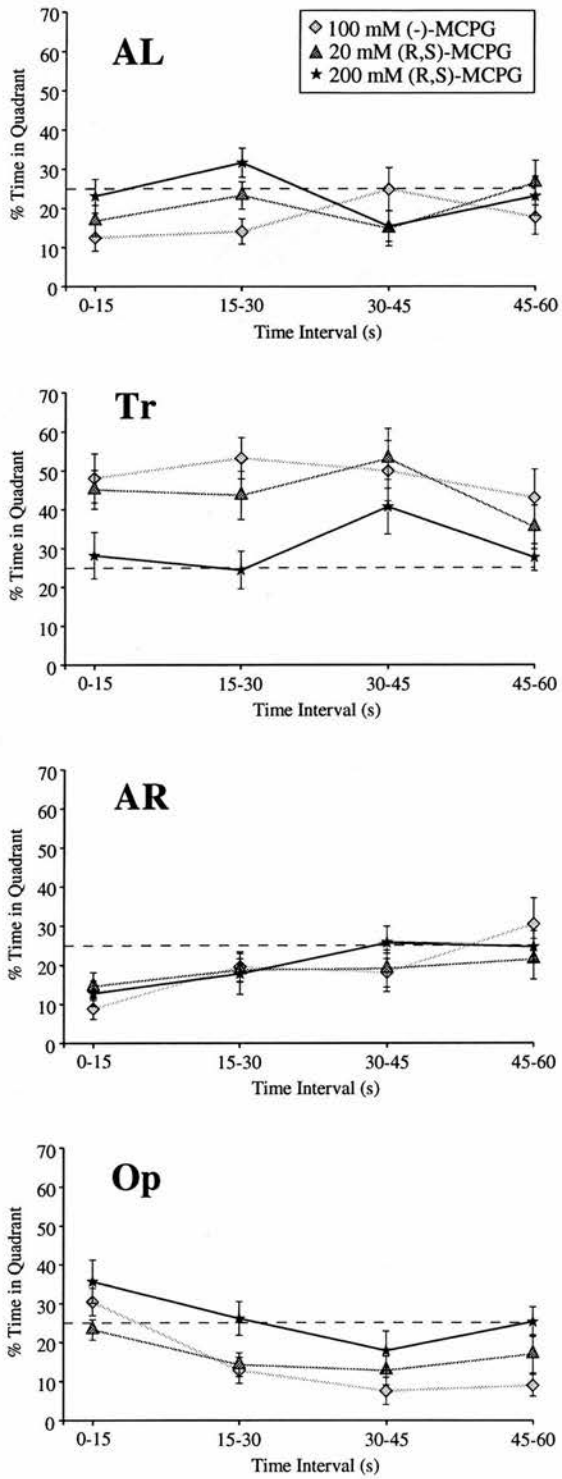


Fig. 6.2.8

Transfer test performance broken down into four 15 s time intervals Data for each quadrant are displayed separately (AL = adjacent left, Tr = training quadrant, AR = adjacent right, Op = opposite).

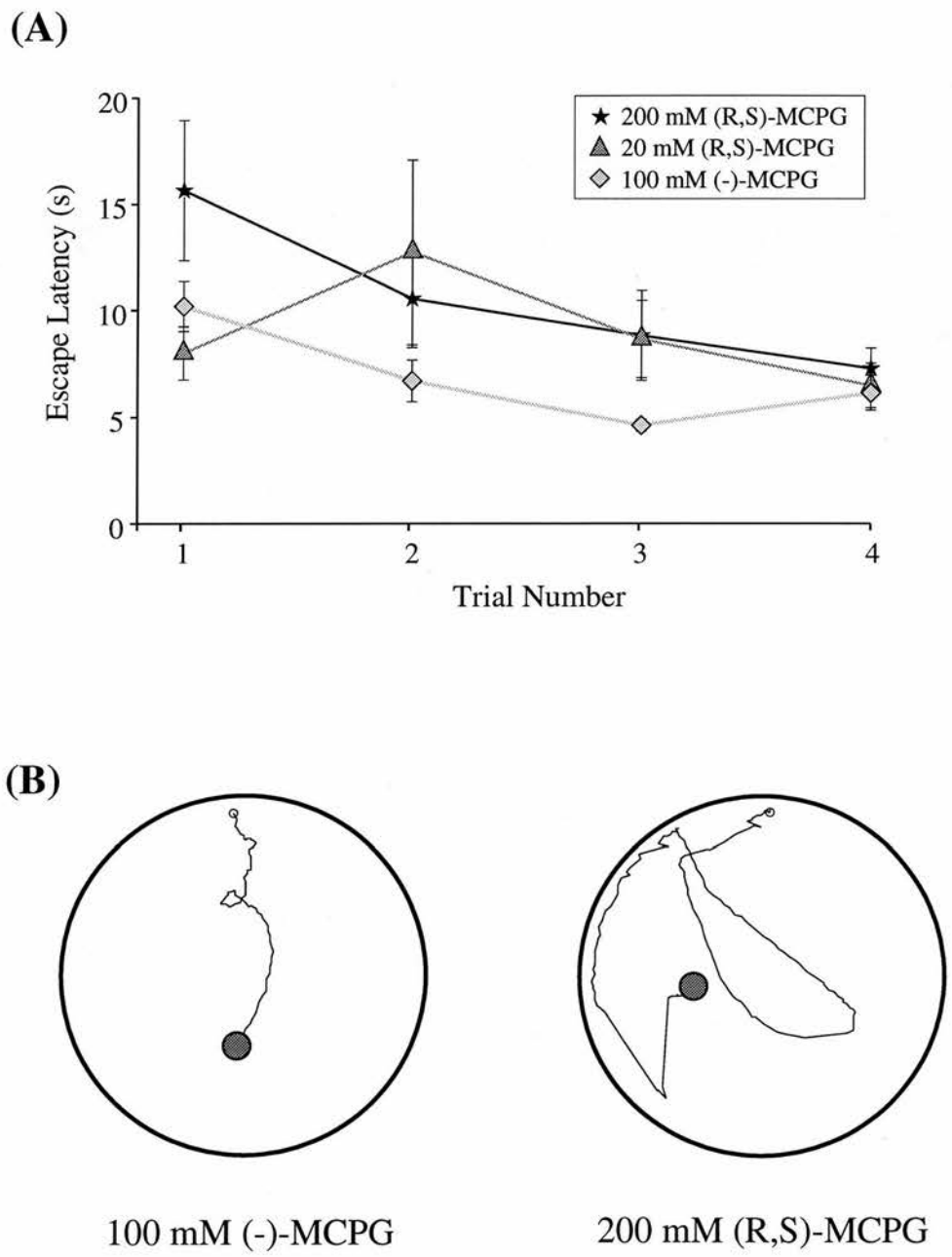


Fig. 6.2.9

(A) Latency to reach the visible escape platform. Rats infused with 200 mM (R,S)-MCPG were significantly impaired relative those infused with (-)-MCPG. (B) Sample swim paths taken on trial 1 of visible platform testing. A subset of rats treated with 200 mM (R,S)-MCPG, such as the example shown here, took longer than 20 s to find the platform.

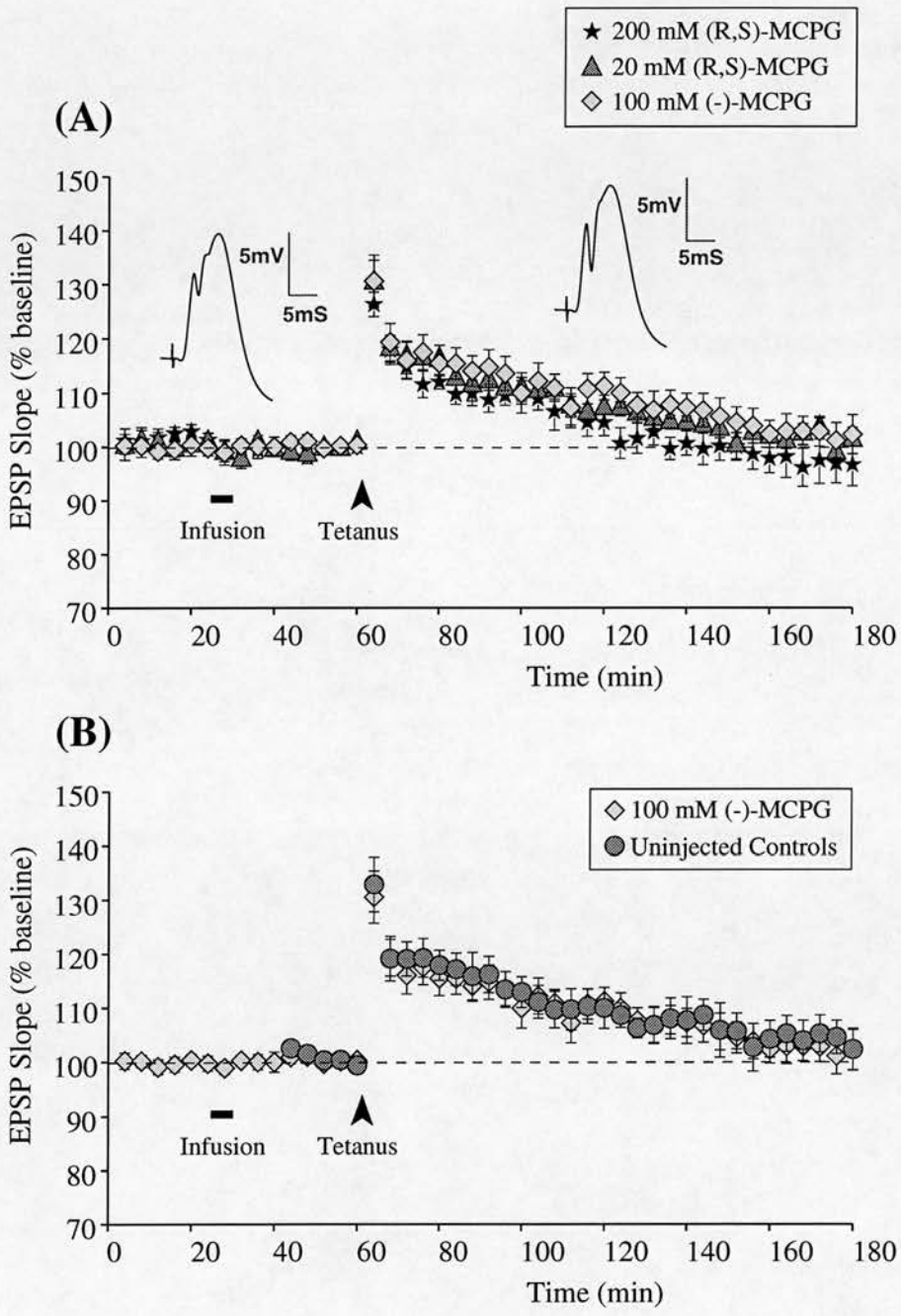


Fig 6.2.10

(A) EPSP slope potentiation, measured for 2 hr after tetanization. Lasting LTP was not induced in any group. (B) The failure to induce LTP was not due to the infusion of (-)-MCPG, since equivalent results were obtained with uninfused controls.

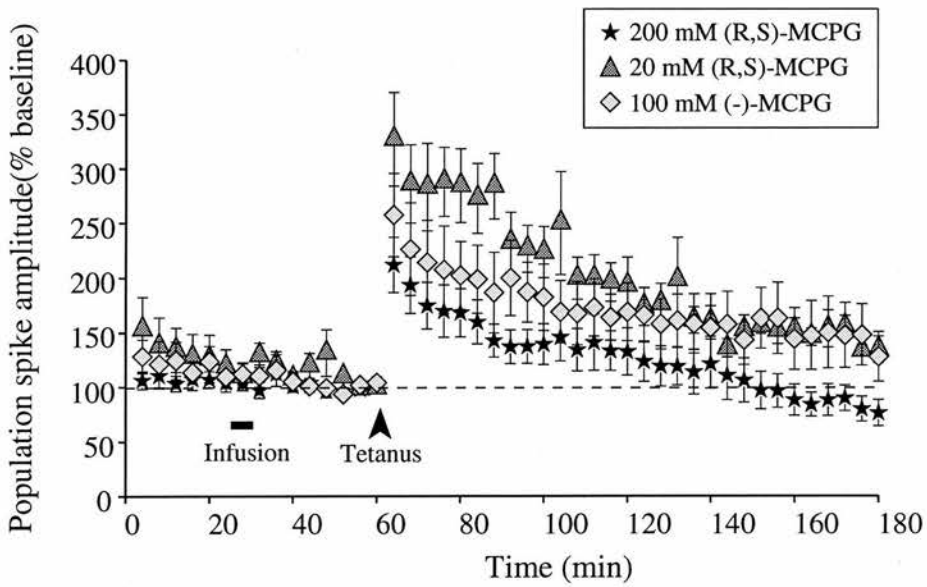


Fig. 6.2.11

Infusion of 200 mM (R,S)-MCPG appears to limit the induction of population spike LTP. However, very little lasting LTP was induced in rats infused with (-)-MCPG or 20 mM (R,S)-MCPG, and the group difference did not reach significance, even 2 hr after tetanization.

6.3 Chronic minipump infusion of (*R*,*S*)-MCPG has no effect on spatial reference memory

6.3.1 Introduction

It was originally planned to carry out a watermaze matching-to-place task using MCPG, since the finding of a delay-dependent deficit on such a task would provide strong evidence for genuine a role of mGluRs in learning. However, it was first decided to test animals chronically infused with MCPG via osmotic minipumps, a procedure which has been used many times in this laboratory in the past, and is considerably more convenient than the delivery of acute drug infusions prior to each training trial.

6.3.2 Methods

6.3.2.1 Surgery

Osmotic minipumps were implanted as described in chapter 4.4.1. Minipumps contained either 100 mM (*R*,*S*)-MCPG (n = 10) or aCSF (n = 10). Rats were allowed 4 days to recover before the start of pre-training, throughout which they were handled and weighed daily.

6.3.2.2 Drugs

A 100 mM solution of (*R*,*S*)-MCPG was prepared as described in experiment 6.2, except that aCSF was used as the vehicle.

6.3.2.3 Behavioural testing

The protocol for reference memory training is detailed in chapter 4.2.2. However, rats were trained to platform positions either in the NW or SE quadrants of the pool, rather than NE and SW. In addition, a second transfer test was carried out 6 days after the first in order to investigate the longer term retention of spatial information during continuous drug infusion. The visible platform task was carried out on the same day as the second transfer test.

6.3.2.4 Histology

Histological verification of cannula sites was carried out as described in chapter 4.5.

6.3.3 Results

6.3.3.1 Acquisition training

Latencies to reach the hidden platform are shown in figure 6.3.1A. The overall reduction in escape latencies over training was highly significant [$F(17,306) = 7.47$; $p < 0.0001$], but the two groups did not differ [$F < 1$].

6.3.3.2 Transfer tests

Transfer test performance is shown in figure 6.3.2A. An ANOVA of the time spent in different quadrants of the pool during the initial transfer test revealed a significant overall bias towards the training quadrant [$F(3,54) = 56.8$; $p < 0.0001$], but no group by quadrant interaction [$F < 1$]. A separate analysis of the time spent in the training quadrant only failed to reveal a difference between groups [$F < 1$]. Representative sample swim paths recorded during transfer tests are shown in figure 6.3.2B.

No significant forgetting occurred between the first and second transfer tests. MCPG-treated rats again performed as well as controls (data not shown).

6.3.3.3 Visible platform task

Latencies to reach the visible platform are shown in figure 6.3.1B. No main effect of trial was obtained [$F(3,54) = 1.0$; $p > 0.3$] and no group difference [$F(1,18) = 1.77$; $p > 0.2$].

6.3.3.4 Sensorimotor disturbances

There was no evidence of sensorimotor or behavioural abnormalities in rats implanted with minipumps containing MCPG. MCPG-treated rats swam equally fast and were no more thigmotaxic than controls (data not shown).

6.3.3.5 Histology

Cannulae were found to be correctly located in the right lateral ventricle in all cases.

6.3.4 Discussion

Continuous infusion of 100 mM (*R,S*)-MCPG had no noticeable behavioural effects of any kind, whether learning-related or sensorimotor. Similarly, no behavioural abnormalities were revealed upon examination of the rats.

It is possible that the dose of MCPG used was simply too low. The Alzet 2000 osmotic minipumps used in the present study infuse continuously for 14 days at 0.5 µl / hr. Hence, a dose equivalent to the 5 µl of 200 mm MCPG used in experiment 6.3 was delivered every 20 hr. However, the relative hippocampal concentrations resulting from these two modes of delivery are unknown, and depend critically on the rate at which MCPG leaves the brain and / or is metabolized.

According to Davis et al. (1992). implantation of minipumps containing 30 mM D-AP5 results in a hippocampal tissue concentration of 0.27 $nmol.mg^{-1}$ wet weight. Subsequent studies have reported concentrations two-fold or even four-fold higher than this (D. M. Bannerman, PhD thesis; Steele and Morris, 1999). However, taking the lowest figure as a guide, and assuming that 1 mg is equivalent to 1 ml, a 30 mM D-AP5 minipump results in a hippocampal concentration of roughly 0.3 mM D-AP5, i.e. a 100-fold reduction in concentration. The pharmacokinetics of MCPG binding and diffusion *in vivo* are unknown, but (*R,S*)-MCPG has a similar molecular weight to D-AP5 (209.2 compared to 197.1). Assuming that the two drugs are comparable, a 100 mM MCPG minipump should result in a tissue concentration of approximately 1 mM. This figure is double the concentration routinely used to block LTP in CA1 slices (see chapter 3.10.3), and as mentioned above, may be a substantial underestimate.

Nevertheless, the assumption that MCPG and AP5 can be treated as equivalent in the above calculation is almost certainly wrong. It has been found that a substantial amount of AP5 is unavailable to NMDA receptors owing to a "trapping" phenomenon (Davis et al., 1992). It is not known whether MCPG behaves in the same way, but if so, the true extracellular concentration of MCPG could be a fraction of that suggested above. In the absence of a reliable assay for the presence of MCPG, these issues remain unresolved.

Assuming that adequate hippocampal MCPG levels were achieved in both cases, the ability of acute but not chronic infusions of MCPG to impair watermaze performance might be due to receptor up-regulation in the latter case. It has been reported that chronic infusion of NMDA receptor antagonists causes an increase in the number of NMDA receptors, but no changes in affinity (Mennini et al.,

1994). It is possible that changes in either affinity or number of mGlu receptors during chronic infusion of MCPG may have compensated for the presence of the antagonist, and resulted in unimpaired performance. Note that minipumps were implanted 5 days before the start of spatial acquisition training, allowing considerable time for receptor up-regulation to occur. Such changes may not have been induced after the single acute infusions of MCPG delivered in experiment 6.2.

If the above suggestion is correct, MCPG will only impair learning if delivered acutely. However, the intraventricular route is not ideal owing to the range of brain structures reached in addition to the hippocampus, and hence the possibility of sensorimotor side-effects such as the slight impairment in the visible platform task observed in experiment 6.2. In order to overcome these problems, it was decided to train rats in the same reference memory task, but to deliver MCPG acutely via intrahippocampal cannulae.

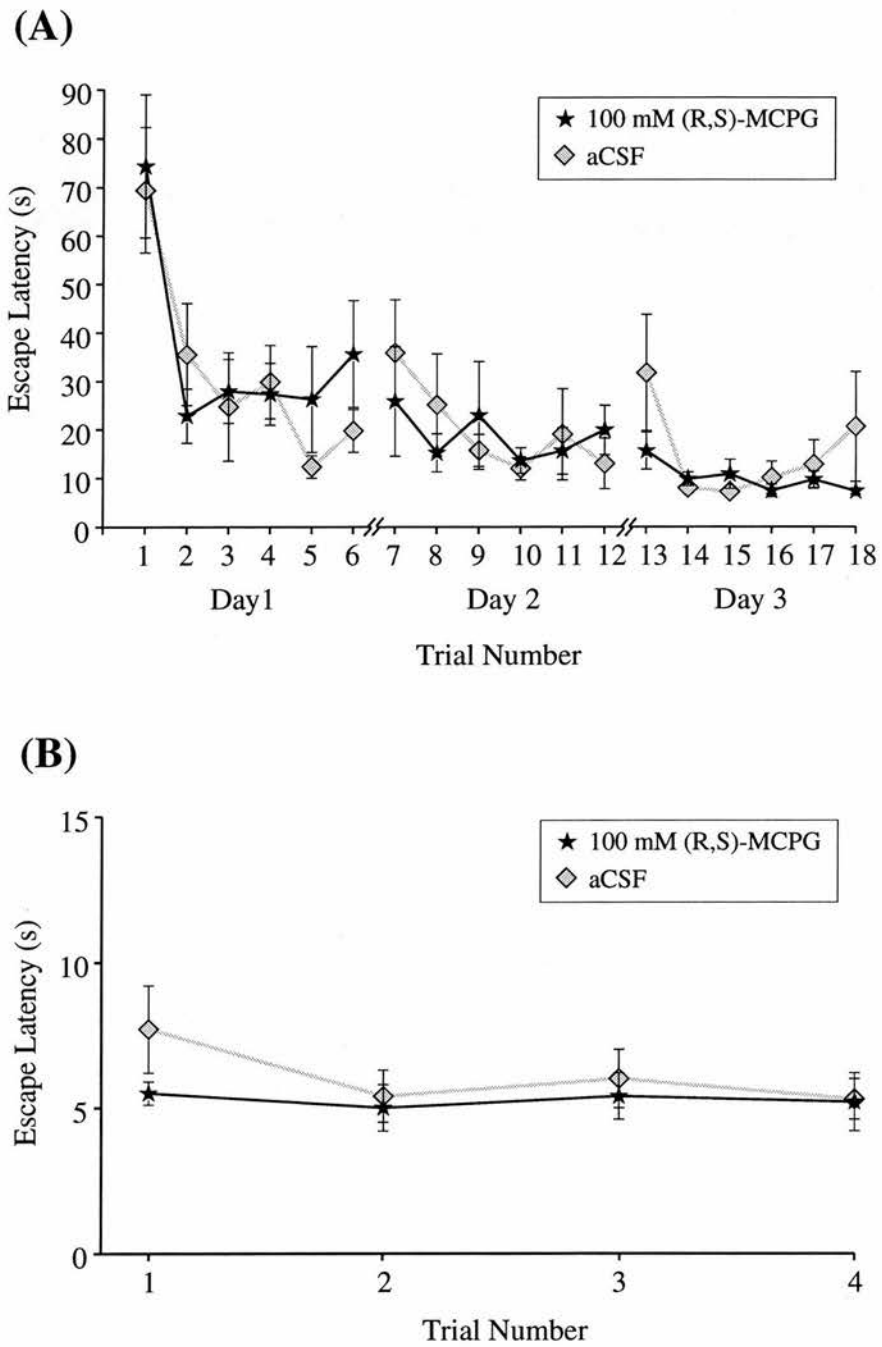


Fig. 6.3.1

Mean latency to find the escape platform during both hidden (A) and visible platform training (B). Rats implanted with minipumps containing 100 mM (R,S)-MCPG performed equally well as controls.

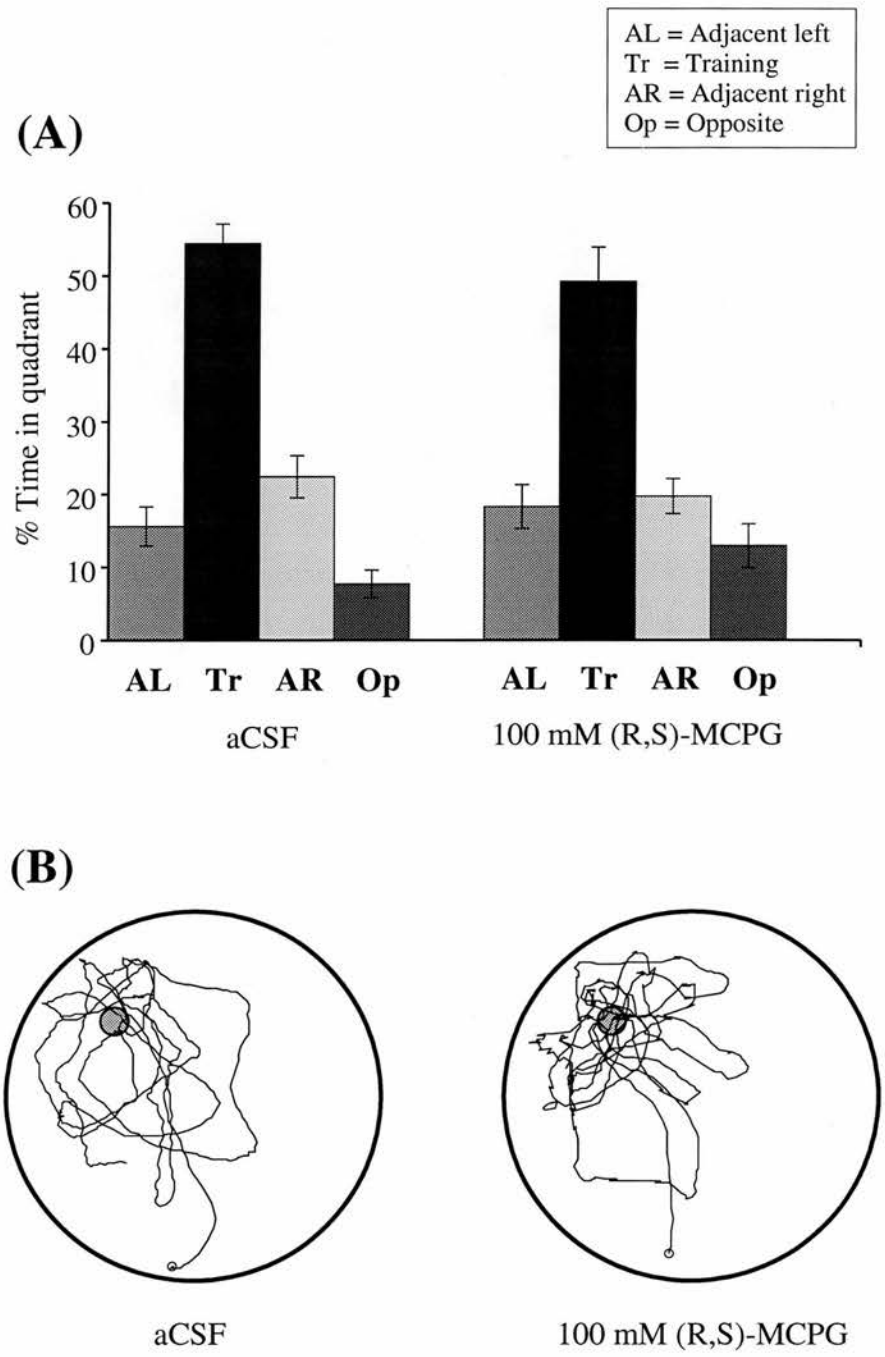


Fig. 6.3.2

(A) Mean percentage time spent within each quadrant of the pool during the transfer test. MCPG-treated rats spent an equal amount of time in the training quadrant to controls. (B) Sample swim paths of rats treated with aCSF or MCPG.

6.4 Acute intrahippocampal infusion of (*R*,*S*)-MCPG has no effect on spatial reference memory.

6.4.1 Methods

6.4.1.1 Surgery

Bilateral intrahippocampal cannulae were implanted as described in chapter 4.4.3. Rats were allowed 1-2 weeks to recover before the start of pre-training, throughout which they were handled and weighed daily.

6.4.1.2 Drugs

A 200 mM solution of (*R*,*S*)-MCPG was prepared as described in experiment 6.3. Rats were infused with 1µl aCSF (n = 10) or 1 µl (*R*,*S*)-MCPG (n = 10).

State dependent and performance control conditions were not included in the present study. Hence, rats were infused with the same drug throughout acquisition training and the transfer test.

6.4.1.3 Behavioural testing

Reference memory training was carried out as detailed in chapter 4.2.2. Pre-training was carried out without prior infusion. Spatial acquisition training, transfer tests, and visible platform training were started 30 min after the end of drug infusion. The visible platform task was carried out 4 days after the transfer test.

6.4.1.4 Histology

The histological procedures used are described in chapter 4.5. The co-ordinates of infusion sites were identified with the aid of a stereotaxic atlas of the rat brain (Paxinos and Watson, 1998).

6.4.2 Results

6.4.2.1 Acquisition training

Latencies to reach the hidden platform are shown in figure 6.4.1A. The overall reduction in escape latencies over training was highly significant [$F(17,306) = 11.8$; $p < 0.0001$], but the two groups did not differ [$F < 1$].

6.4.2.2 Transfer test

Transfer test performance is shown in figure 6.4.2A. Although performance was worse than that obtained in experiments 6.2 and 6.3, an ANOVA of the time spent in different quadrants of the pool revealed a highly significant overall bias towards the training quadrant [$F(3,54) = 6.26$; $p < 0.01$], but no group by quadrant interaction [$F < 1$]. A separate analysis of the time spent in the training quadrant only failed to reveal a difference between groups [$F < 1$]. Representative sample swim paths recorded during the transfer test are shown in figure 6.4.2B.

6.4.2.3 Visible platform task

Latencies to reach the visible platform are shown in figure 6.4.1B. The overall improvement across trials was significant [$F(3,54) = 4.04$; $p < 0.05$], but no difference was found between groups [$F < 1$].

6.4.2.4 Sensorimotor disturbances

4 of the rats infused with MCPG exhibited a slight ataxia and failure to groom when examined 1-2 hr after drug infusion. These symptoms were not evident at the time of behavioural testing, and no abnormal behaviours such as falling off the platform were observed. MCPG-treated rats were no more thigmotaxic than controls, and swam equally fast throughout training (data not shown).

6.4.2.5 Histology

Figure 6.4.3 shows infusion sites for the 20 rats included in the above analysis, All infusion sites were located within the hippocampus. In some cases, hippocampal damage extended for several hundred μm below the site of the guide cannula (see below). In these cases, the infusion site was estimated based on guide cannula position.

6.4.2.6 Relationship between histology and watermaze performance

In 8 rats (3 aCSF and 5 MCPG), hippocampal damage extended ventrally as far as the wall of the ventricular layer situated beneath the inferior blade of the dentate gyrus. Of the 5 MCPG-treated rats in which such damage was observed, 3 exhibited sensorimotor disturbances.

In order to assess any possible impact on behaviour, both aCSF and MCPG groups were sub-divided according to whether damage extended as far as the ventricular wall or not ("intact" or "damaged" groups). Figure 6.4.4 shows the percentage time spent in the training quadrant during the transfer test re-analysed in this way. Rats infused with aCSF performed equally well, regardless of the extent of hippocampal damage. However, MCPG-infused rats in the "damaged" group performed at chance, whilst those in the "intact" group performed as well, or better than controls, with no hint of the slight mean impairment evident in the overall analysis (figure 6.4.2A). A modest impairment in escape latencies on the final day of acquisition training was also observed in the MCPG "damaged" group (data not shown). Despite the above findings, MCPG-treated rats in the "damaged" condition were normal in terms of thigmotaxis, swimming speed, and performance in the visible platform task (data not shown).

However, owing to the small and uneven group sizes, a statistical analysis of the above data was not attempted, and any attempt to draw conclusions from these findings is necessarily speculative.

6.4.3 Discussion

In contrast to the effects of acute intraventricular infusion of MCPG, acute intrahippocampal infusion does not cause a deficit in watermaze performance. As in experiment 6.3, it is possible that the dose of MCPG used was simply too low. However, the concentration used is close to the solubility limit of MCPG. This problem might be overcome by continuous intrahippocampal minipump infusion of MCPG, but if receptor up-regulation occurs during chronic infusion as suggested in experiment 6.3, this method of drug administration would be unlikely to produce an impairment.

It is interesting that a deficit was observed in MCPG-treated rats whose hippocampal damage extended ventrally as far as the wall of the ventricular layer that links the dorsal third ventricle with the lateral ventricles. It is possible that MCPG was not limited to the hippocampus in these cases, but leaked into the ventricles to some extent, thus mimicking the effects of i.c.v. infusion. MGPG-treated rats in the "damaged" group showed a similar watermaze deficit to that obtained after acute ventricular infusion in experiment 6.2. Possible reasons for the success of acute i.c.v. infusions, but the failure of acute intrahippocampal infusions are suggested in section 6.5.

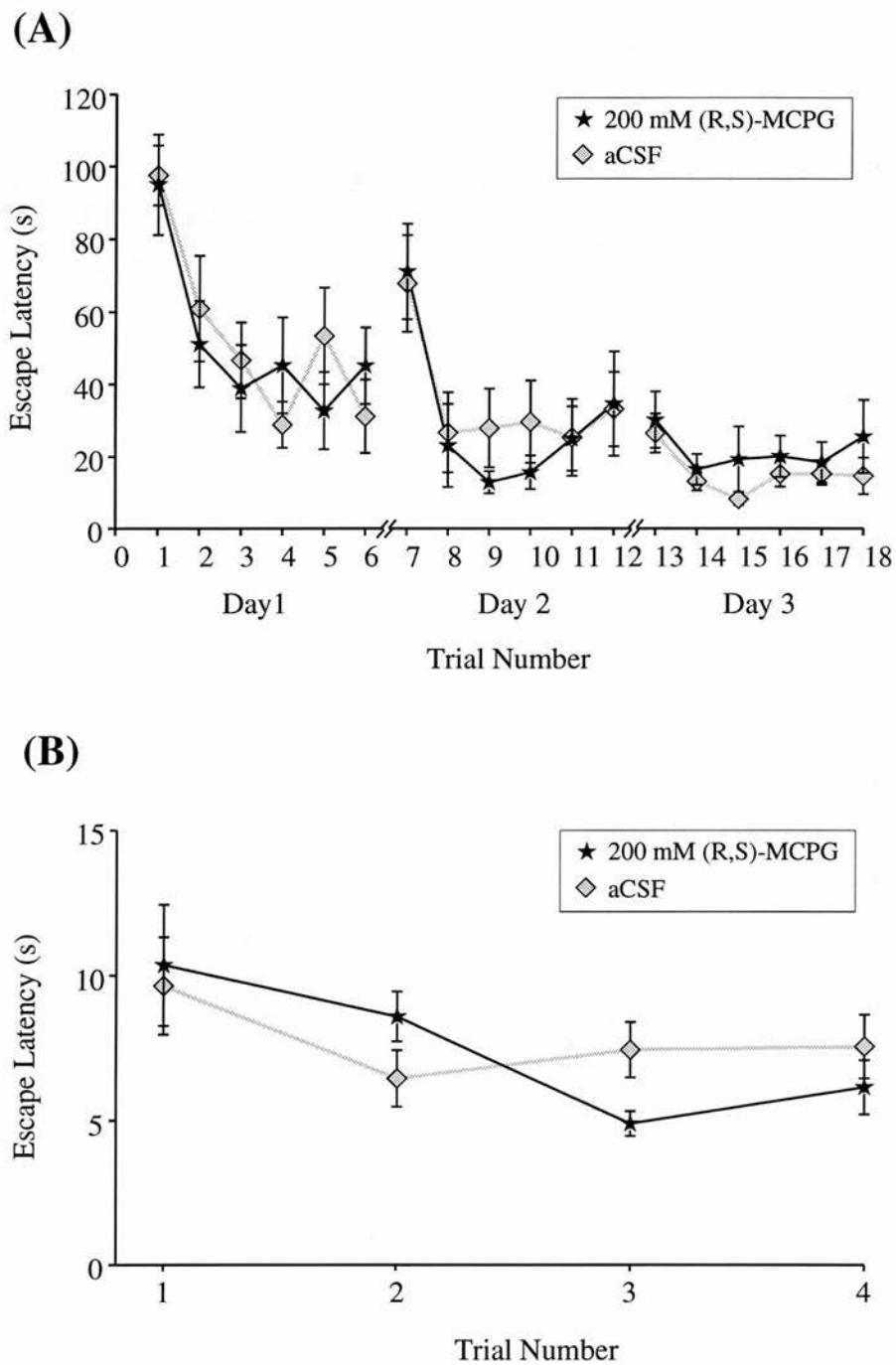


Fig. 6.4.1

Mean latency to find the escape platform during both hidden (A) and visible platform training (B). Rats given intrahippocampal infusions of 200 mM (R,S)-MCPG performed equally well as controls.

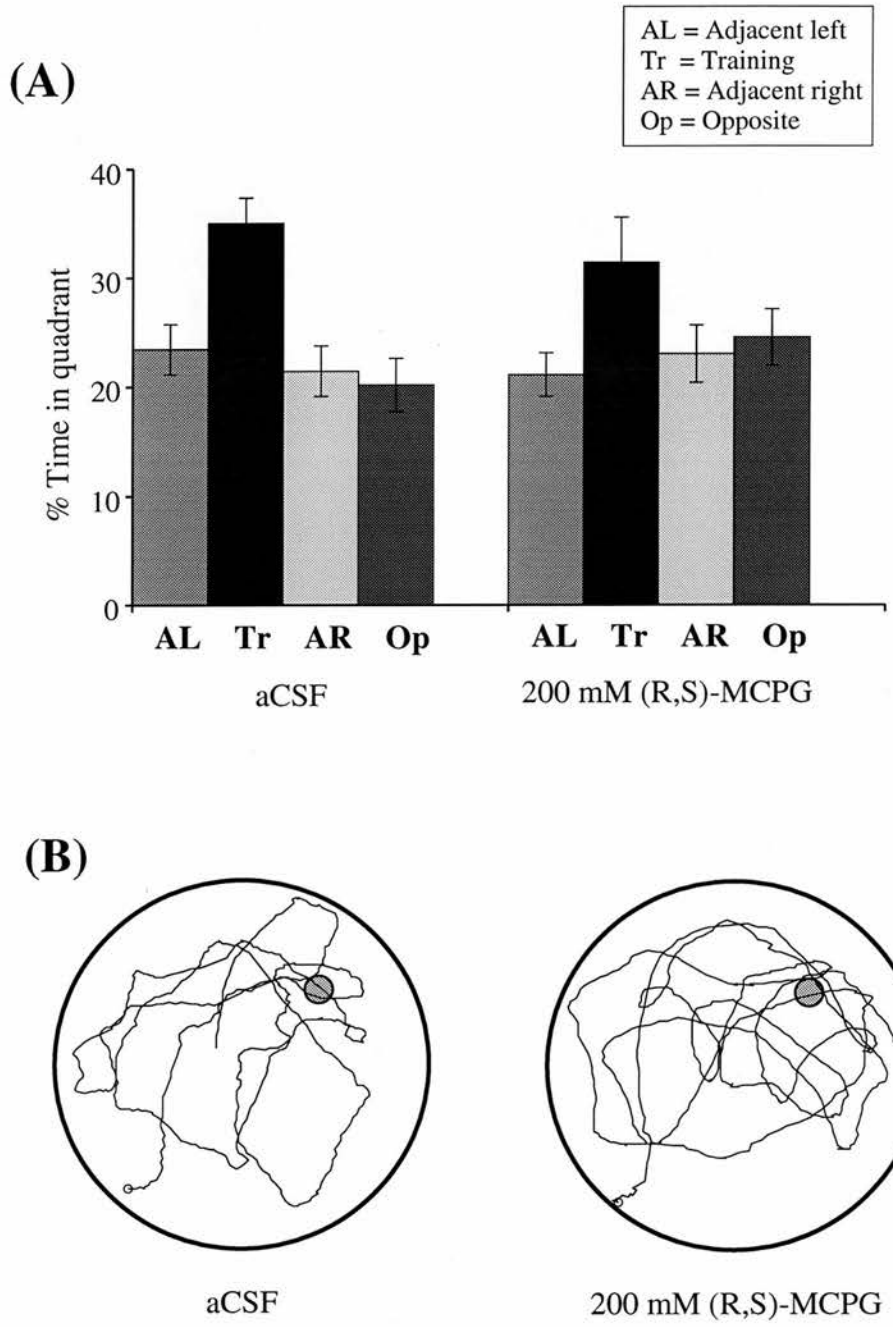


Fig. 6.4.2

(A) Mean percentage time spent within each quadrant of the pool during the transfer test. MCPG-treated rats spent an equal amount of time in the training quadrant to controls. (B) Sample swim paths of rats treated with aCSF or MCPG.

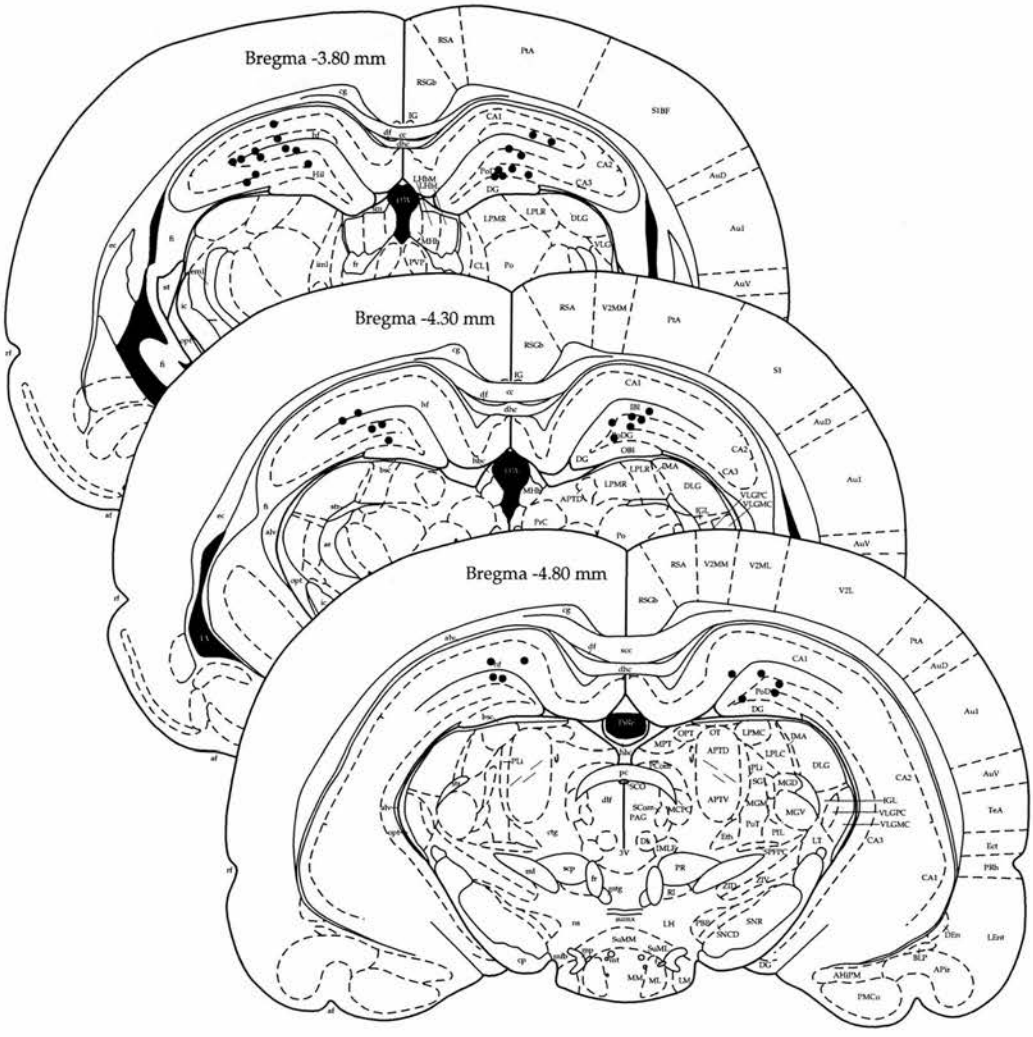


Fig. 6.4.3
Estimated intrahippocampal infusion sites for all rats tested in experiment 6.3 (n = 20).

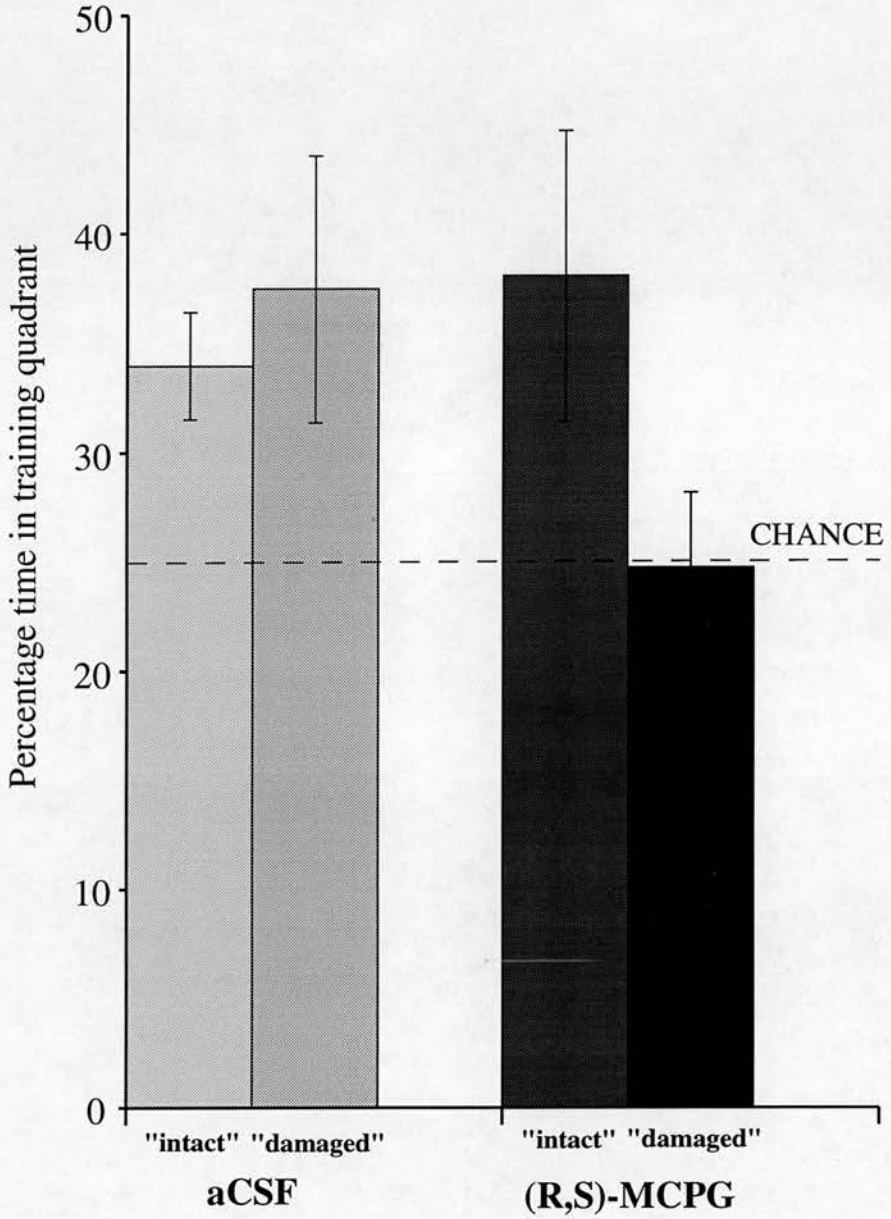


Fig. 6.4.4

Percentage time spent within the training quadrant during the transfer test sub-divided into "intact" and "damaged" groups (see text).

6.5 General discussion

The results of experiment 6.2 are broadly consistent with those of existing reports (see section 6.1), i.e. i.c.v. infusion of MCPG causes a watermaze deficit that is almost exclusively limited to the transfer test. However, experiment 6.3 revealed that chronic infusion of MCPG by the same route has no effect on performance. Surprisingly, the same was found to be true of acute intrahippocampal infusion (experiment 6.4). Nevertheless, there was an indication in this last experiment that MCPG-treated rats were impaired when hippocampal damage extended ventrally towards the ventricular layer, suggesting that in these cases, infusion may have been effectively more intraventricular than intrahippocampal.

There are at least two alternative explanations for the success of acute i.c.v. infusions of MCPG, but the failure of acute intrahippocampal infusions. Firstly, MCPG may induce a watermaze deficit by actions on structures other than the hippocampus. Secondly, intrahippocampal infusions may lead to blockade of mGluRs within a very limited portion of the hippocampus, whilst leaving sufficient drug-free tissue for normal task performance. These possibilities are discussed below.

This first possibility is consistent with the fact that MCPG causes a deficit in the percentage time spent within the target quadrant during the transfer test, but little or no deficit in latency to reach the platform during acquisition training, and no deficit in swimming initially to the correct location in the transfer test. It is true that transfer test performance is a more sensitive and less variable measure of spatial learning than escape latency. However, a transfer test is essentially an extinction trial, during which persistent searching is required in the absence of reward. It is possible, for example, that a decreased motivation to escape resulting from an anxiolytic action of MCPG outside of the hippocampus might result in a selective transfer test deficit owing to the requirement for persistent searching, as well as spatial memory.

Nevertheless, such an interpretation is hard to reconcile with the finding that rats infused with vehicle throughout training were not impaired when given MCPG for the first time during the transfer test in experiment 6.2. However, this result is based on a very small sample (n = 4). It is worth noting that Bordi et al. (1996) reported a modest but significant deficit in this condition using a larger sample of rats (n = 9). Hence, it is possible that the "learning" impairment seen after i.c.v. infusion of MCPG is in fact a performance deficit, perhaps caused by the blockade of mGluRs outside the hippocampus. This interpretation is also consistent with the finding in experiment 6.2 that acute i.c.v. infusion of MCPG causes a slight impairment on a visually cued control task.

As discussed in section 5.1.1, intraventricular infusion of a drug results in a fairly widespread distribution of drug throughout the forebrain. It is likely that i.c.v. infusion of MCPG in experiment

6.2 resulted in appreciable blockade of mGluRs in many brain areas, including the thalamus, striatum, and frontal cortex. Thalamic mGluRs, in addition to NMDA receptors, are known to be involved in the transmission of sensory information (Salt and Eaton, 1996), and hence the blockade of mGluRs in this structure might be expected to cause a performance deficit, rather than a genuine learning impairment.

mGluRs are also highly expressed in the striatum, a structure reported to play a role in watermaze place learning (Devan et al., 1996). The blockade of striatal mGluRs results in the inhibition of long-term depression *in vitro* (Calabresi et al., 1993), although no studies of the role of striatal mGluRs in spatial learning have been conducted. The medial prefrontal cortex is another region that has been implicated in spatial learning, and in which mGluRs are expressed. MCPG has been found to block LTP in isolated slices of medial frontal cortex (Vickery et al., 1997). The role of frontal mGluRs in spatial learning has not been studied, but medial prefrontal cortex lesions have been reported to impair the acquisition of a spatial reference memory task in the watermaze (Kolb et al., 1982). However, others have failed to find such a deficit on acquisition, but report an impairment of spatial reversal learning (deBruin et al., 1994). This latter finding is consistent with other reports that the medial prefrontal cortex is necessary for successful watermaze navigation under changing task conditions (Granon and Poucet, 1995; Compton et al., 1997). The above discussion illustrates the fact that there are a number of different ways in which intraventricular MCPG infusion might impair watermaze performance, in addition to its actions in the hippocampus.

The second possibility mentioned earlier concerns the likelihood that a single point infusion of MCPG will result in a limited distribution of the drug along the septotemporal axis of the hippocampus. It has been reported that an island of dorsal hippocampal tissue comprising only 26 % of the total volume is sufficient for normal performance of a watermaze reference memory task (Moser et al., 1995). Hence, the blockade of mGluRs within a restricted region of the dorsal hippocampus might be insufficient to impair learning. This problem is not likely to occur after ventricular drug infusion, since the hippocampus is surrounded by ventricular structures, allowing access to its entire septotemporal length. However, the superior distribution of drug within the hippocampus is achieved at the cost of allowing substantial quantities of drug to enter surrounding structures.

Unfortunately, the data obtained in the present series of experiments cannot distinguish between the two possibilities discussed above. A pessimistic conclusion, however, might be that MCPG is a questionable choice of mGluR antagonist for use in such tasks. MCPG is a rather weak mGluR antagonist with an uncertain profile of subtype selectivity (see chapter 3.6.3). It produces a modest watermaze impairment when injected intraventricularly at the highest concentration possible, and then only under certain circumstances. It is likely that antagonists with considerably greater potency and subtype selectivity than MCPG will be necessary to produce an unambiguous resolution of all the

issues raised in the present study. Watermaze experiments involving the preferential group I mGluR antagonist AIDA are currently underway.

Chapter Seven

The effect of the mGluR antagonist (*R,S*)-α-methyl-4-carboxyphenylglycine (MCPG) on dentate LTP *in vivo*.

7.1 General introduction

The effect of the putative group I and II mGluR antagonist MCPG on hippocampal NMDA receptor-dependent LTP is currently a controversial topic, and the literature contains several conflicting reports (see chapter 3.10). At the time when the current study was carried out, three laboratories had published reports concerning the ability of MCPG to block LTP *in vivo*.

Riedel and colleagues found that unilateral i.c.v. infusion of 200 mM (*R,S*)-MCPG 30 min before tetanization completely blocked both STP and LTP in freely moving rats. A lower dose of 20 mM (*R,S*)-MCPG limited the duration of LTP to 2–3 hr (Riedel and Reymann, 1993; Riedel et al., 1995a). It was subsequently reported that continuous perfusion of the dentate with 5 mM (*R,S*)-MCPG via a push-pull cannula limited the duration of LTP to 2 hr in urethane-anaesthetized rats. Acute bilateral i.c.v. infusion of 10 µl (*R,S*)-MCPG (5 µl per ventricle) 40 min before tetanization reduced the magnitude of LTP, but did not cause a total block (Richter-Levin et al., 1994). However, LTP was recorded for less than 1 hr after tetanization in this part of the study. Despite these positive results, it was reported elsewhere that unilateral i.c.v. infusion of 5 µl 200 mM (*R,S*)-MCPG had no effect on LTP in urethane-anaesthetized rats (Bordi and Ugolini, 1995).

The reasons for the discrepancies in results obtained in different laboratories are unknown (see chapter 3.10.5 for discussion). The experiments conducted below were carried out in order to determine, initially, whether or not a blockade of LTP with MCPG could be obtained in this laboratory (experiments 7.3 and 7.4), and whether MCPG reverses the electrophysiological effects of the mGluR agonist (1*S*,3*R*)-ACPD (experiment 7.5).

One possible explanation for the negative results obtained in many laboratories concerns the position of the mGluR-activated molecular switch described by Bortolotto et al. (1994). Hence, additional experiments were conducted in order to address the possibility that an mGluR-activated molecular switch must be reset before a block of LTP with MCPG can be demonstrated (see Bortolotto et al., 1994; Wang et al., 1995; but also Selig et al., 1995; Thomas and O'Dell, 1995; experiment 7.6).

7.2 General methods

The procedures described below constitute methods that were common to all experiments in the current series, but not covered in chapter 4. Aspects of the methodology unique to a particular experiment will be introduced later, as necessary.

7.2.2 Drugs

Two different concentrations of (*R,S*)-MCPG were tested: 200 mM and 20 mM. Solutions were prepared as described in chapter 6.2.1.2, except that phosphate buffered saline (PBS) was used as the vehicle. Despite the high concentration of NaOH present in the 200 mM (*R,S*)-MCPG solution, the pH was found to be 7.28, i.e. very nearly neutral. A 100 mM stock solution of D-2-amino-5-phosphopentanoate (D-AP5) was made up in equimolar NaOH. Spiking with small aliquots (1-2µl) of 5M NaOH was sometimes required to ensure that the drug dissolved fully. A final concentration of 30 mM was attained by dilution with the appropriate volume of aCSF. Solutions were stored at -20 °C in small aliquots before use.

7.2.3 Electrophysiology

Experiments were conducted under urethane anaesthesia as described in chapter 4.3. In addition, two stainless steel injection cannulae, connected by plastic tubing to microinfusion syringes, were lowered into the left and right lateral ventricles (AP = -0.9 mm; Lat = ± 1.3 mm; DV = -4.5 mm from the skull surface). Miniature thermistors (Fenwell Electronics Ltd.) were glued to the end of a length of insulated copper tubing, such that the total diameter of the thermistor and shaft was approximately 0.5 mm (although early attempts were slightly thicker). Each thermistor was calibrated by measuring the resistance of the device (in arbitrary units recorded by computer) whilst the tip was immersed to a depth of approximately 3 mm in a beaker of water. The water was stirred constantly (using a magnetic stirrer), and its temperature, as determined with a mercury thermometer, was varied over an appropriate range. An example of the relationship between thermistor reading and temperature thus derived is shown in figure 7.2. The equation describing the best linear fit to these data was used, off-line, to convert arbitrary thermistor readings into temperature expressed in degrees centigrade. In each rat, a thermistor was lowered into the right dentate gyrus (AP = -3.5 mm; Lat. = 2.0 mm; DV = 3.0 mm from dura), and thermistor readings were sampled every 20 s for the duration of an experiment.

Throughout the experiment, perforant path test stimulation consisted of biphasic pulses of 100 µs

half-pulse duration delivered at 0.05 Hz, except during input / output curves when pulses were delivered at 0.1 Hz.

Half an hour or more after implantation of electrodes a number of test pulses were delivered and the stimulation intensity was adjusted to elicit a population spike of 2-4 mV. At this point, a test baseline was recorded. Once potentials had stabilized (typically 30-60 min later), the main experiment was started. High frequency tetanization consisted of 5 trains of 20 pulses at 250 Hz, with 10s between trains; the stimulation intensity was set at twice the baseline level.

According to linear fit:

Thermistor reading = 2979.0 - (41.85 x temperature)
=> Temperature = (thermistor reading - 2979.0) / -41.85

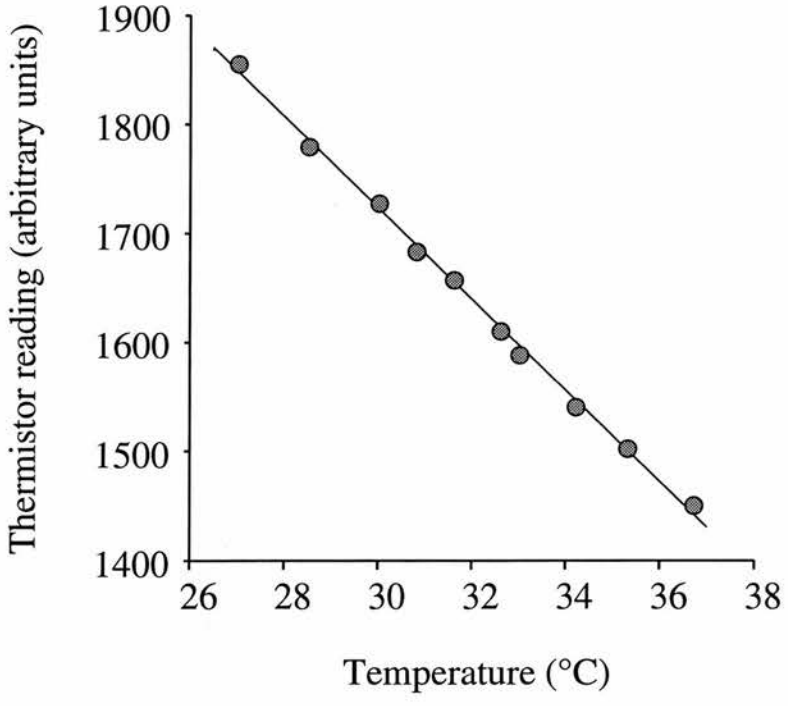


Fig. 7.2

Sample thermistor calibration data. Thermistor readings were converted into temperature data according to the equation described above.

7.3 Does MCPG block dentate LTP in vivo?

7.3.1 Methods

Once potentials assessed during the test baseline period had been stable for at least 20 min, an input / output (I / O) curve consisting of 4 pulses at each of 10 stimulation intensities over the range 0.1-1.0 mA was recorded. Immediately afterwards, a 20 min baseline period was started, followed by a 10 µl bilateral drug infusion (i.e. 5 µl per ventricle) of either 200 mM (*R,S*)-MCPG (n = 7), 20 mM (*R,S*)-MCPG (n = 6), or vehicle (n = 7), delivered over a period of 10 min. Half an hour after the end of the infusion, a second, partial, I / O curve, consisting of 4 pulses at 200 µA, 400 µA, and 600 µA only, was recorded. Following this, animals received a high frequency tetanus. Field potentials were recorded for a further 2 hr after tetanization. Finally, a third I / O curve was recorded over the full intensity range.

In order to test the drug infusion system, a further control group was infused with 30 mM D-AP5 (n = 6). In two of these animals, stimulation and recording electrodes were implanted bilaterally; only one pathway received a high-frequency tetanus.

7.3.2 Results

7.3.2.1 EPSP slope potentiation

Figure 7.3.1A shows the amount of EPSP slope potentiation in the three main groups, normalized to the mean value obtained during the 10 min prior to tetanization; neither dose of MCPG caused a block of LTP. Sample EPSPs recorded before and after LTP induction in a vehicle-infused rat are illustrated. Separate ANOVAs of the mean slope potentiation over different 10 min time periods revealed no significant group differences in EPSP slope LTP 50-60 min or 110-120 min post-tetanus; no group differences were found in percentage PTP (table 7.3.1). No significant group differences in EPSP slope LTP were observed at any time point after tetanization when the results were re-normalized to the 10 min prior to drug infusion, rather than tetanization (data not shown).

Table 7.3.1 EPSP slope potentiation normalized to pre-tetanus baseline

	EPSP slope PTP 0-4 min post-tetanus (% baseline)	**EPSP slope LTP 50-60 min post-tetanus (% baseline)**	**EPSP slope LTP 110-120 min post-tetanus (% baseline)**
Vehicle	144.2 ± 2.7	123.3 ± 2.4	116.9 ± 2.4
20 mM (*R,S*)-MCPG	146.9 ± 3.6	121.8 ± 3.5	116.3 ± 4.6
200 mM (*R,S*)-MCPG	143.7 ± 2.7	119.9 ± 3.2	109.9 ± 4.0
ANOVA results	$F < 1$	$F < 1$	$F(2,17) = 1.21$; $p > 0.3$

7.3.2.2 Effect of D-AP5 on EPSP slope potentiation

Infusion of 10 µl 30 mM D-AP5 caused a complete block of LTP. Figure 7.3.1B shows pooled EPSP slope data from the 6 rats infused with AP5 compared to the vehicle-infused controls. Mean non-tetanized control pathway data from the two rats in which recordings were made bilaterally are plotted alongside data from the six tetanized pathways. Curiously, infusion of AP5 caused a slight increase in EPSP slope, an effect noticeable in both tetanized and control pathways, and associated with a transient temperature rise of approximately 0.5 °C (see below). However, the elevated pre-tetanus slope magnitude appears to be maintained throughout the experiment. Inspection of figure 7.3.1B suggests that this is most likely due to the superposition of an EPSP slope increase induced by AP5, upon a slight chronic baseline rise. (A similar slight baseline rise was found in later experiments: see figure 7.4.1A, control pathways.) Nevertheless, equivalent changes were seen in both tetanized and non-tetanized pathways. In the two rats which received bilateral stimulation and recording, no differences were found between tetanized and non-tetanized EPSP slope values 2 hr after tetanization, relative to the pre-tetanus baseline, indicating that LTP was completely blocked (tetanized pathways 110-120 min post tetanus: 109.7 % and 103.0 %; corresponding non-tetanized pathways: 111.3 %; 98.1 %). Hence, in these two animals at least, the possibility that a residual potentiation was masked by baseline changes can be ruled out.

7.3.2.3 EPSP slope potentiation across the I / O curve

Figure 7.3.2 shows the mean EPSP slope I / O curves for all groups. Data are normalized to the maximum EPSP slope value recorded during the first I / O curve, prior to drug infusion and tetanization. The initial and final I / O curves, which span the full range of stimulation intensities, are

plotted as line graphs. The three points of the shortened I / O curve carried out immediately prior to tetanization are superimposed upon the full I / O curves. The proximity of these points to the corresponding points on the pre-infusion I / O curve suggest that drug-induced baseline changes were minimal, even in the 200 mM (*R,S*)-MCPG group, when assessed in this way (but see discussion).

Inspection of figure 7.3.2 suggests that there is little difference in the potentiation induced across the I / O curve in the three drug groups. This was examined more formally by measuring the percentage potentiation occurring between the initial and final I / O curves at each test pulse intensity (i.e. [initial value / final value] x 100). The results of this analysis are shown in figure 7.3.3. An ANOVA in which stimulation intensity was entered as a within subject factor revealed no significant overall group difference [$F < 1$], or group x stimulation intensity interaction [$F < 1$], indicating that the slight reduction in LTP seen in the 200 mM (*R,S*)-MCPG group was not significant. The result also confirms that the failure to find a block of LTP with MCPG is not an artifact of the test pulse stimulation intensities chosen for sampling during the main experiment.

7.3.2.4 Population spike potentiation

No significant differences in the percentage population spike potentiation were found at any time point after tetanization (figure 7.3.4; table 7.3.2).

Table 7.3.2 Percentage population spike potentiation.

	Population spike PTP: amplitude 0-4 min post-tetanus (% baseline)	**Population spike LTP: amplitude 50-60 min post-tetanus (% baseline)**	**Population spike LTP: amplitude 110-120 min post-tetanus (mV)**
Vehicle	511.8 ± 207.9	449.7 ± 213.5	330.7 ± 138.8
20 mM (*R,S*)-MCPG	388.3 ± 11.6	298.4 ± 30.8	231.1 ± 32.2
200 mM (*R,S*)-MCPG	430.4 ± 26.2	347.6 ± 67.7	250.3 ± 55.6
ANOVA results	$F < 1$	$F < 1$	$F < 1$

However, the percentage increase in population spike amplitude is dependent on the initial baseline amplitude. This can lead to misleading results especially if baseline values fall to very low levels prior to tetanization, as sometimes happened in the present experiment. For instance, the large standard

errors in the vehicle group are due largely to a baseline fall in one animal, leading to an artificially high value for population spike LTP. Under these circumstances, it is more appropriate to compare absolute changes in amplitude. Accordingly, the mean absolute spike amplitude over the 10 min prior to tetanization was subtracted from the spike amplitude at each individual time point, resulting in a measure of population spike potentiation which is independent of the initial baseline value (figure 7.3.5A). Nevertheless, no significant group differences in the mean absolute population spike increase were found at any time point after tetanization (table 7.3.3)

Table 7.3.3 Absolute population spike potentiation.

	Increase in population spike amplitude 0-4 min post-tetanus (mV)	**Increase in population spike amplitude 50-60 min post-tetanus (mV)**	**Increase in population spike amplitude 110-120 min post-tetanus (mV)**
Vehicle	4.76 ± 0.69	3.60 ± 0.76	2.51 ± 0.58
20 mM (*R,S*)-MCPG	5.03 ± 0.36	3.46 ± 0.36	2.25 ± 0.48
200 mM (*R,S*)-MCPG	4.25 ± 0.53	3.10 ± 0.29	1.81 ± 0.48
ANOVA results	$F < 1$	$F < 1$	$F < 1$

7.3.2.5 Effect of D-AP5 on population spike LTP

Infusion of 30 mM D-AP5 caused a dramatic fall in population spike amplitude (figure 7.3.5B), far greater than that predicted by the modest temperature rise induced by D-AP5 infusion (see below). Furthermore, the fall in population spike amplitude is far more rapid in onset than the temperature rise. This effect of AP5 has been noted in previous studies *in vivo* (Errington et al., 1987; Abraham and Mason, 1988; Bordi and Ugolini, 1995) and has been attributed to the influence of an extrinsic afferent pathway (Abraham and Mason, 1988). However, data from the two non-tetanized control pathways reveal that the population spike depression was transient, lasting little more than 1 hr after drug infusion. Figure 7.3.5B shows the absolute increase in population spike amplitude following D-AP5 infusion and tetanization, normalized to the 10 min prior to drug infusion owing to the baseline changes described above. Data from vehicle-infused controls, analysed in the same way, are shown for comparison. Infusion of D-AP5 caused a complete block of population spike LTP.

7.3.2.6 Baseline stimulation parameters

The mean absolute slope of the pre-infusion baseline field EPSP and population spike amplitude recorded over the 10 min prior to drug infusion did not differ across groups; no significant group differences in stimulation intensity were found (table 7.3.4).

Table 7.3.4 Baseline parameters.

	Stimulation Intensity (μA)	**Pre-infusion EPSP slope (mV/ms)**	**Pre-infusion population spike amplitude (mV)**
Vehicle	318.6 ± 39.8	3.61 ± 0.33	2.51 ± 0.20
20 mM (*R,S*)-MCPG	291.7 ± 24.1	3.61 ± 0.28	2.92 ± 0.23
200 mM (*R,S*)-MCPG	282.9 ± 39.7	3.48 ± 0.37	2.46 ± 0.18
ANOVA results	$F < 1$	$F < 1$	$F(2,17) = 1.55$; $p > 0.2$

7.3.2.7 Changes in brain temperature

Figure 7.3.6A shows brain temperature changes throughout the experiment normalized to the mean temperature over the 10 min before infusion. An ANOVA of the mean temperature rise over the 30 min after drug infusion revealed a significant effect of group [$F(2,17) = 6.04$; $p < 0.05$]. *Post hoc* Newman-Keuls pairwise comparisons revealed significant temperature rises in both the 20 mM and 200 mM (*R,S*)-MCPG groups relative to vehicle-infused controls (table 7.3.5). However, these increases rarely exceeded 0.5 °C, and no significant group differences were seen over the final 30 min of the experiment [$F < 1$; table 7.3.5].

No significant differences were found in the absolute baseline brain temperature recorded over the 10 min prior to drug infusion (table 7.3.5). The difference between these values and the body temperature maintained by the heating blanket (36.2 °C) is most likely due to heat loss through the burr hole made to allow insertion of the thermistor, as well as through the surrounding skull. The non-significant trend towards a group difference is explained by the slightly lower initial temperature recorded in the vehicle group. However, this difference is an artefact of thermistor design: the thermistor used for the first three vehicle-injected rats consistently gave lower temperature readings than those used subsequently. This thermistor was slightly thicker in diameter than later ones (see section 7.2.3), and is likely to have caused a greater amount of damage upon insertion.

Table 7.3.5 Temperature increase caused by MCPG infusion.

	Pre-infusion brain temp. (°C)	**Mean increase in brain temp. during 30 min after infusion (°C)**	**Mean increase in brain temp. during final 30 min of recording (°C)**
Vehicle	32.34 ± 0.80	0.01 ± 0.09	0.14 ± 0.18
20 mM (*R,S*)-MCPG	33.59 ± 0.16	0.47 ± 0.14	0.35 ± 0.33
200 mM (*R,S*)-MCPG	34.16 ± 0.42	0.30 ± 0.04	0.46 ± 0.11

ANOVA results	$F(2,17) = 2.80$; p > 0.05	$F(2,17) = 6.04$; $p < 0.02$	$F < 1$

Figure 7.3.6B shows the temperature increase induced by infusion of 30 mM D-AP5, normalized to the 10 min prior to drug infusion. Data from vehicle-infused rats are shown for comparison. An ANOVA of the mean temperature change over the 30 min following drug infusion in vehicle and AP5 groups revealed that the temperature increase caused by AP5 infusion was significant (table 7.3.6).

Table 7.3.6 Temperature increase caused by D-AP5 infusion.

	Pre-infusion brain temp. (°C)	**Mean increase in brain temp. during 30 min after infusion (°C)**	**Mean increase in brain temp. during final 30 min of recording (°C)**
Vehicle	32.24 ± 0.80	0.01 ± 0.09	0.14 ± 0.18
30 mM D-AP5	30.95 ± 0.10	0.34 ± 0.12	0.31 ± 0.31

ANOVA results	$F(1,11) = 1.24$; $p > 0.2$	$F(1,11) = 4.95$; $p < 0.05$	$F < 1$

7.3.2.8 Rats excluded from analysis

Data from one member of the 200 mM (*R,S*)-MCPG group were not included in the above analysis. In this rat, the EPSP slope had fallen to less than 70% of the baseline value 2 hr after tetanization; the EPSP amplitude fell by a similar amount. This dramatic fall was never seen in any of the other MCPG-infused rats. At the end of the experiment, the recording electrode was lowered slightly and

the signal recovered to its pre-tetanus level, or slightly above. It is probable that the recording electrode shifted upwards slightly during the course of the experiment.

One rat infused with AP5 and implanted bilaterally with stimulation and recording electrodes was excluded from analysis on the basis of baseline criteria described in experiment 7.4.

7.3.3 Discussion

These results indicate that MCPG does not have any effect on LTP. Nevertheless, although not significant, the mean potentiation after 2 hr was smallest in the 200 mM (*R,S*)-MCPG group. However, inspection of Figure 7.3.1A suggests that the EPSP slope baseline of this group, following a slight rise after the drug infusion (possibly an effect of the small temperature increase described above), was falling throughout the 20 min before tetanization. This decline was slight, and did not result in a reduction in slope potentiation measured 2 hr after tetanization, even when normalized to the pre-infusion, rather than pre-tetanus baseline (data not shown). Nevertheless, a baseline fall was consistently observed in rats infused with 200 mM (*R,S*)-MCPG. This phenomenon may reflect a small metabotropic contribution to normal synaptic transmission. It has been reported that MCPG infusion into the basolateral amygdala causes a transient depression of baseline responses (Keele et al., 1995). Similarly, MCPG perfusion has recently been found to cause a lasting EPSP slope depression in dentate slices (Huang et al., 1997). A gradual chronic baseline fall induced by MCPG might give rise to an apparent block of LTP, particularly when followed for several hours after tetanization. In order to detect and, if necessary, control for such a chronic baseline fall, the above study was repeated, this time using a second, non-tetanized pathway in each animal.

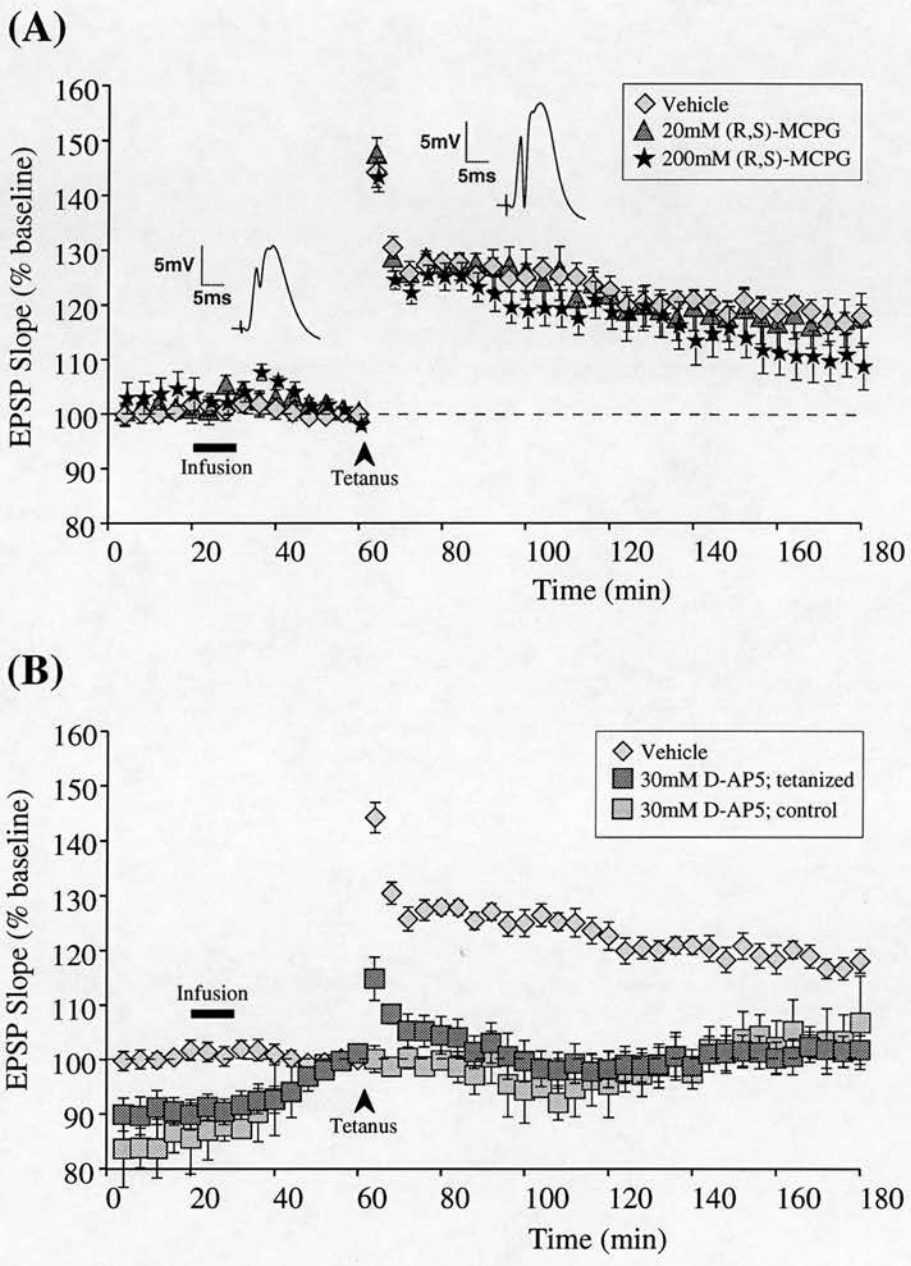


Fig. 7.3.1

(A) Infusion of (R,S)-MCPG did not significantly reduce EPSP slope LTP. Representative sample EPSPs recorded immediately before tetanization and 30 min afterwards are shown. (B) Infusion of D-AP5 caused a complete block of EPSP slope LTP. Data from the 6 animals infused with D-AP5 are plotted against the vehicle-infused control data from figure A. Of the 6 rats infused with D-AP5, 2 underwent bilateral stimulation and recording; mean data from the 2 control pathways are plotted alongside data from the 6 tetanized pathways.

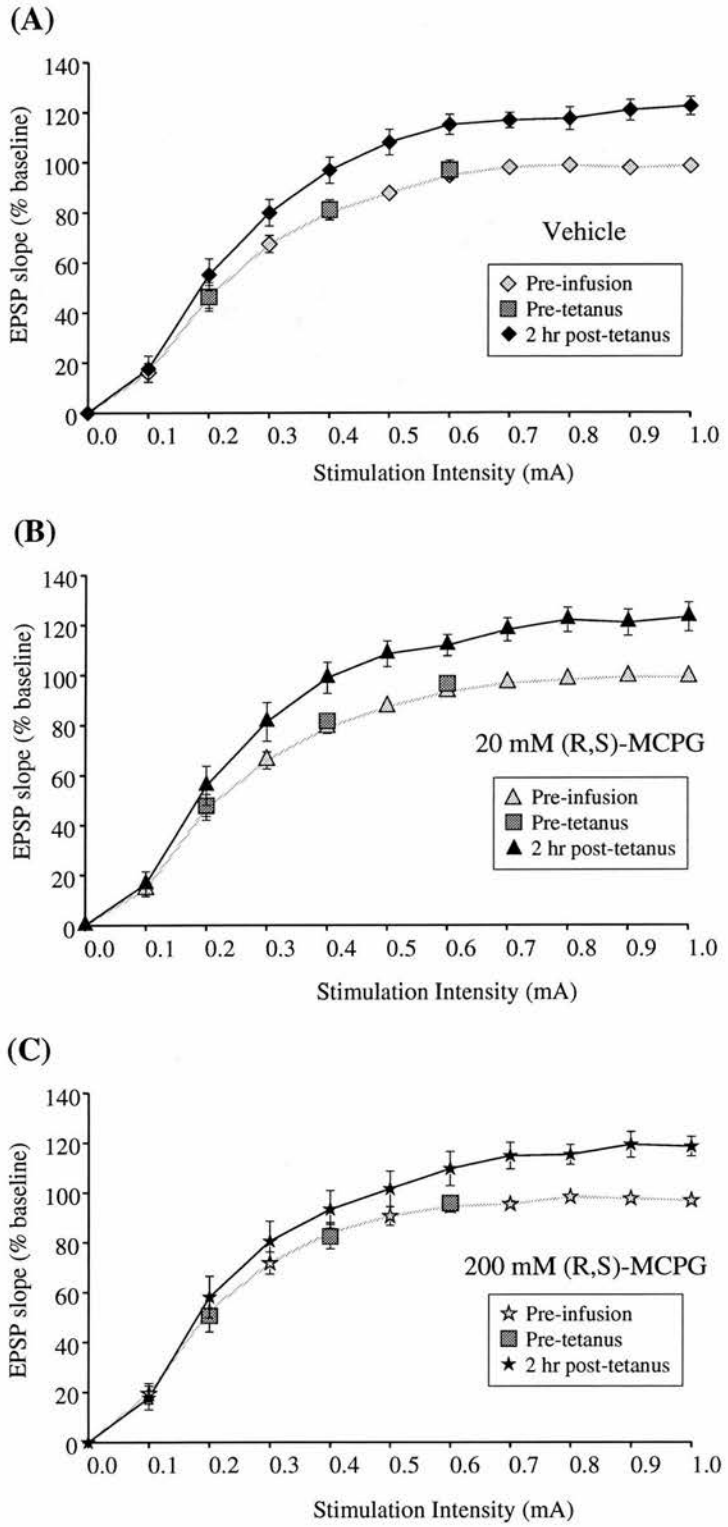


Fig 7.3.2

EPSP slope input / output curves recorded before and after drug infusion and 2 hr after tetanization. Rats treated with 20 mM (R,S)-MCPG (B) or 200 mM (R,S)-MCPG (C) showed equivalent LTP across the I / O curve to rats treated with vehicle (A).

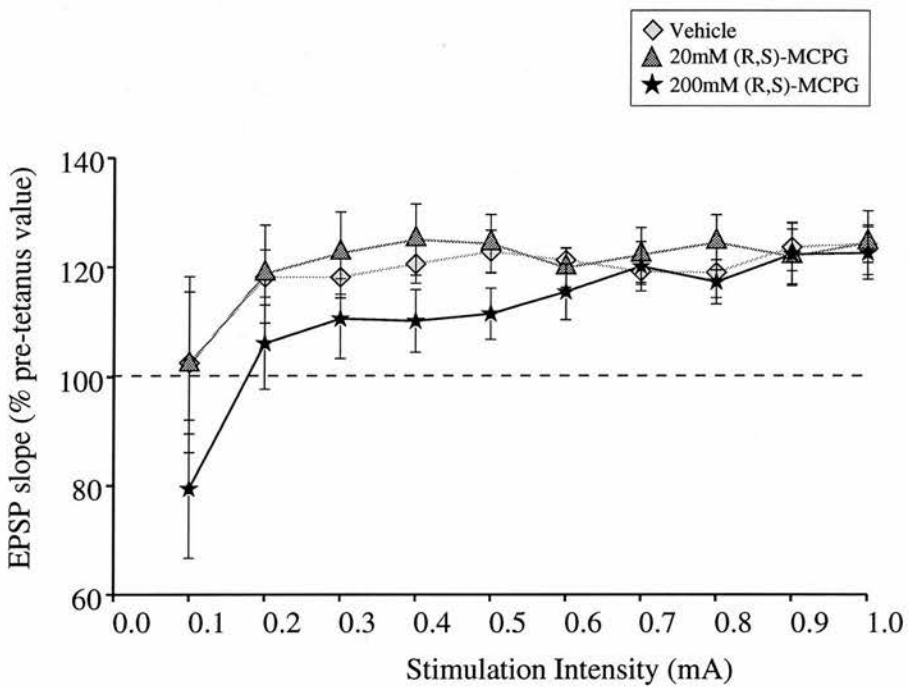


Fig 7.3.3

Percentage increase in EPSP slope before and 2 hr after tetanization measured across the full range of the I/O curve. Infusion of MCPG did not cause a significant reduction in LTP analysed in this way.

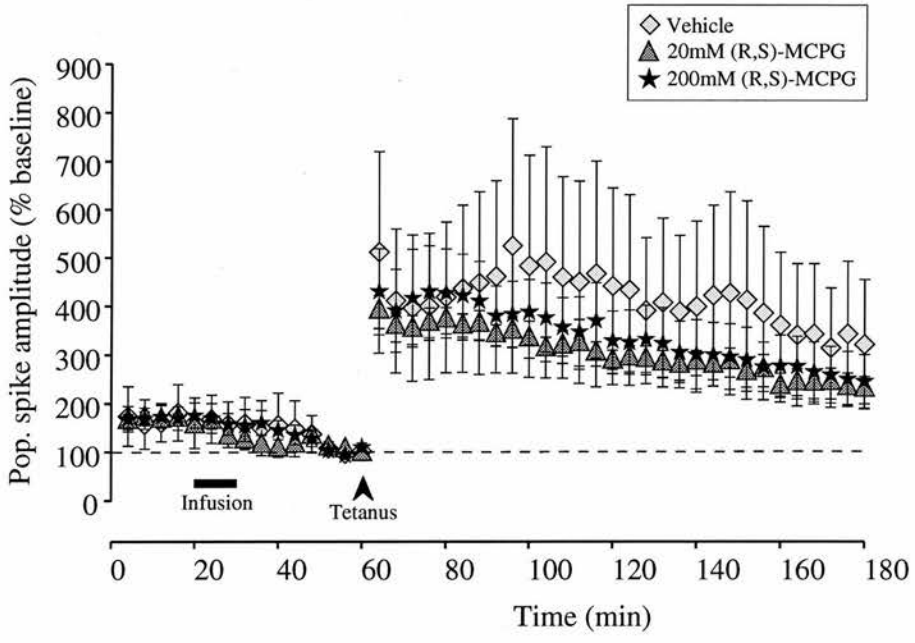


Fig. 7.3.4

Infusion of MCPG did not significantly reduce the percentage population spike potentiation. The large standard errors were caused by falling baselines, which resulted in artificially high percentage increases in some cases (see text for discussion).

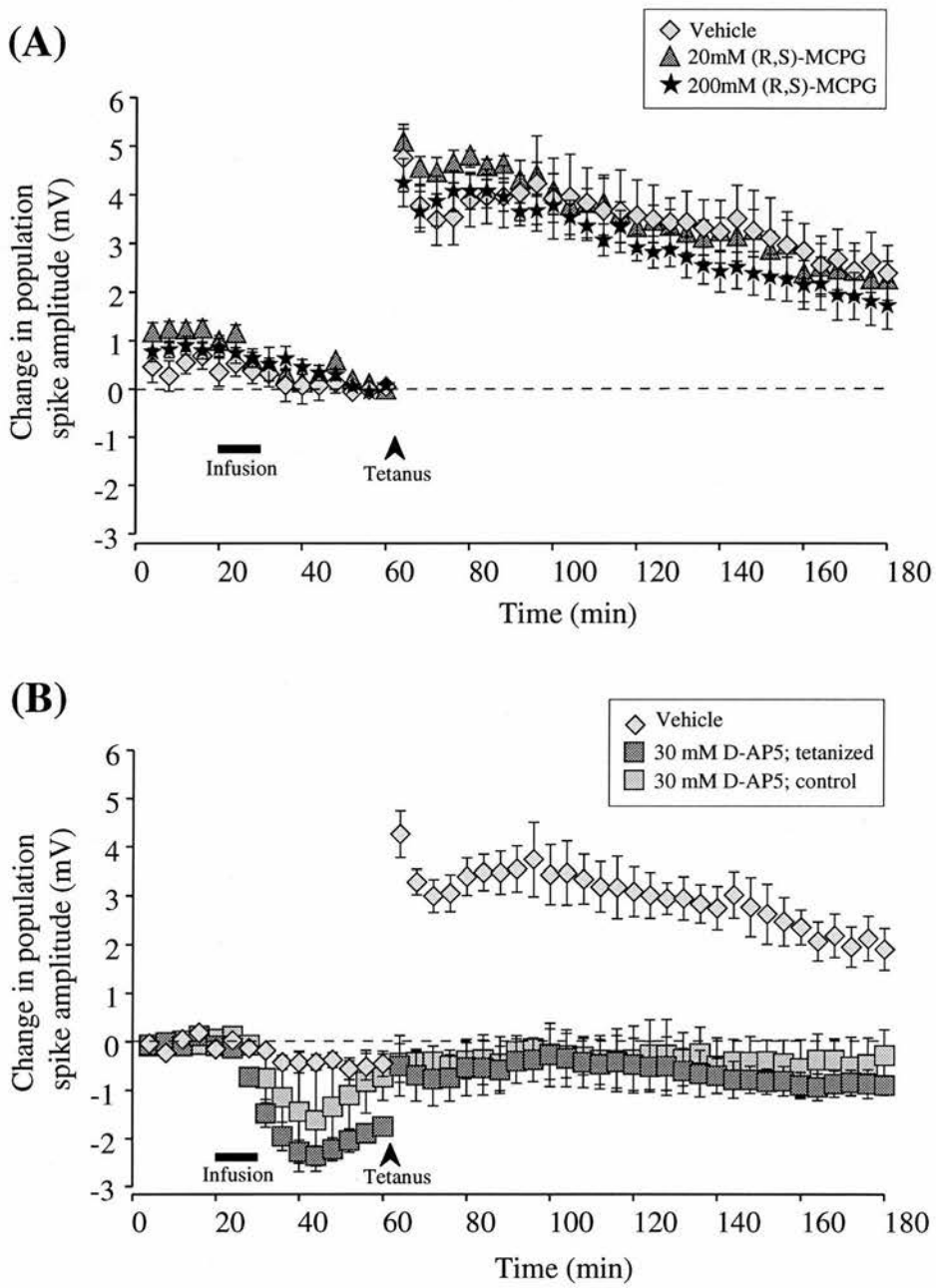


Fig. 7.3.5

(A) An equivalent increase in population spike amplitude was seen in all groups after tetanization. (B) D-AP5 infusion caused a transient fall in population spike amplitude, and LTP was completely blocked relative to vehicle-infused controls. Data are normalized to 10 min prior to infusion owing to the AP5-induced fall in population spike. Mean non-tetanized control pathway data from the 2 rats in which bilateral recordings were made are plotted alongside data from the 6 tetanized pathways.

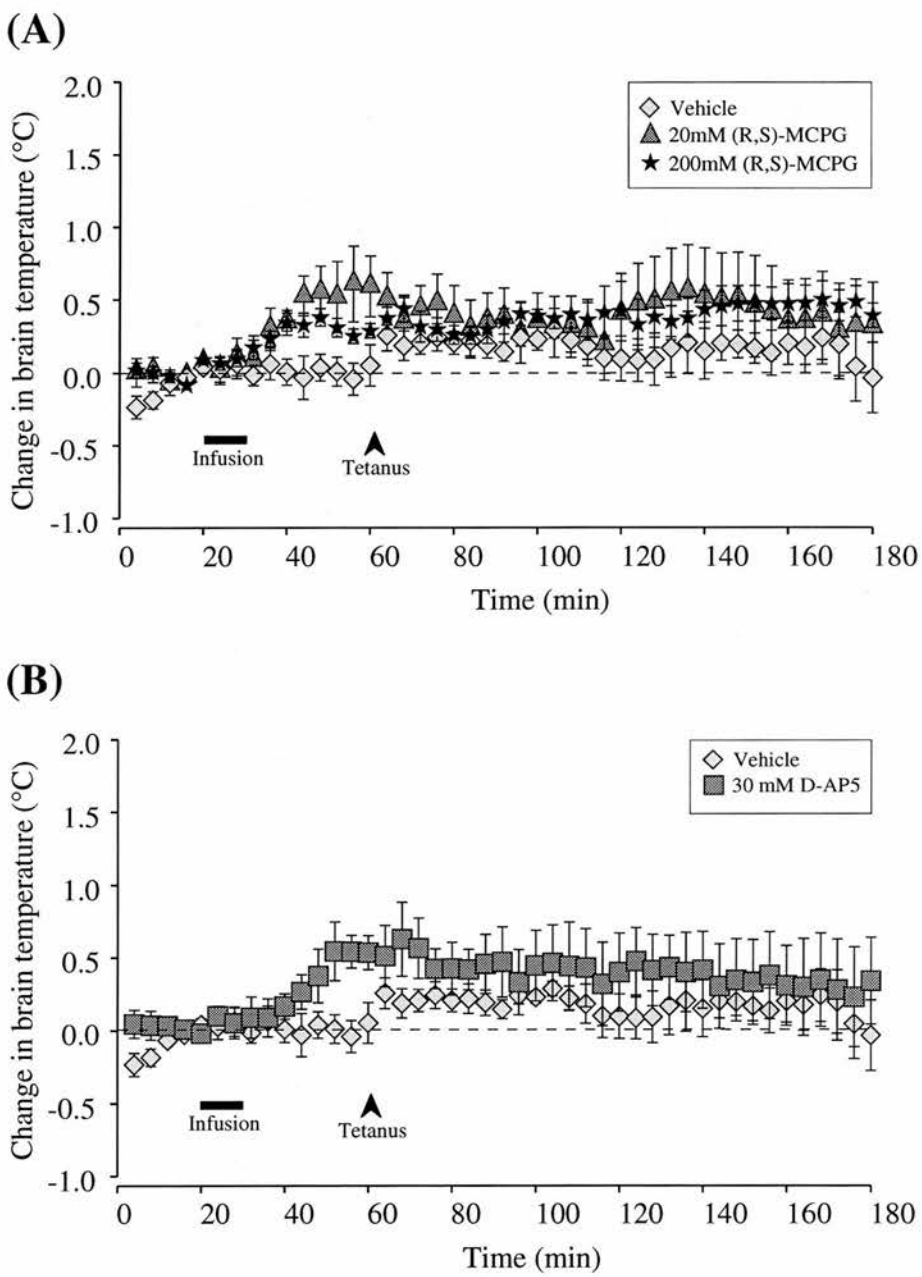


Fig. 7.3.6

(A) Infusion of both 20 mM 200 mM (R,S)-MCPG caused a slight temperature rise relative to vehicle-infused controls. (B) A similar temperature increase was seen after infusion of 30 mM D-AP5.

7.4 Does MCPG block dentate LTP *in vivo*? A two-pathway study

7.4.1 Methods

Most procedures were identical to those described for experiment 7.3. However, stimulating and recording electrodes were positioned in the right hippocampal formation in addition to the left, and the thermistor was positioned approximately 2 mm posterior and 1 mm lateral to the right-hand recording electrode. Test pulse stimulation intensity was adjusted in each pathway to give a population spike of 2-4 mV as before. The two pathways were stimulated alternately, but only the left perforant path was given a high frequency tetanus. Rats received infusions of either 200 mM (*R,S*)-MCPG or vehicle. Data from animals in which a seizure had occurred in either pathway during positioning of the electrodes were discarded. Likewise, if the final normalized slope baseline [(tetanized/control) x100] changed by more than 10 % during the hour before tetanization, the results were not included in the final analysis. Examples of such failed experiments are described in section 7.4.2.7.

7.4.2. Results

7.4.2.1 EPSP Slope LTP

MCPG infusion again failed to block LTP. Figure 7.4.1A shows the data for both control and tetanized pathways, normalized to the mean value ten minutes prior to tetanization (MCPG: n = 5; aCSF: n = 7). Separate ANOVAs at different time points, in which tetanized and control pathway values were entered as sub-groups within each drug treatment group, revealed no significant group by pathway interaction in the amount of PTP or LTP after 1 or 2 hr (see table 7.4.1). The tetanized pathways were still significantly potentiated overall relative to the control pathways 110-120 min post-tetanus [$F(1,20) = 14.2$; $p < 0.01$]. *Post hoc* pairwise comparisons at this time point (Newman-Keuls) revealed that tetanized pathways were significantly potentiated relative to control pathways in both individual groups [200 mM (*R,S*)-MCPG: $p < 0.05$; vehicle: $p < 0.05$]. Pairwise comparisons between tetanized pathways in the 200 mM (*R,S*)-MCPG and vehicle groups failed to reveal a significant group difference 0-4 min, 50-60 min, or 110-120 min post-tetanus. Similarly, no significant group differences were found between non-tetanized control pathways at any of these time points.

As in experiment 7.3, I / O curves were recorded 20 min before drug infusion and 2 hr after tetanization. No difference in the amount of EPSP slope LTP induced in the tetanized pathways of MCPG and vehicle groups was observed at any point on the I / O curve (data not shown).

Table 7.4.1 Two-pathway study: EPSP slope potentiation

	EPSP slope PTP 0-4 min post-tetanus (% baseline)		**EPSP slope LTP 50-60 min post-tetanus (% baseline)**		**EPSP slope LTP 110-120 min post-tetanus (% baseline)**	
	tetanized	**non-tetanized**	**tetanized**	**non-tetanized**	**tetanized**	**non-tetanized**
Vehicle	144.8 ± 5.41	99.8 ± 1.60	117.0 ± 2.5	104.3 ± 4.1	112.5 ± 2.1	102.2 ± 2.7
200 mM (*R,S*)-MCPG	149.8 ± 6.57	102.7 ± 1.52	122.8 ± 2.6	105.4 ± 1.5	116.7 ± 3.7	106.5 ± 3.1

ANOVA results	Drug group x pathway interaction NS [$F < 1$].	Drug group x pathway interaction NS [$F < 1$].	Drug group x pathway interaction NS [$F < 1$].

In figure 7.4.1B the EPSP slope data are plotted using the non-tetanized pathway as a within subject control at each time point throughout the experiment, i.e. normalized value = [(tetanized / control) x 100]. Equivalent LTP was seen in both groups. No significant group differences were found 0-4 min post-tetanus, 50-60 min post-tetanus, or 110-120 min post-tetanus (table 7.4.2).

Table 7.4.2 Two-pathway study: normalized EPSP slope potentiation.

	EPSP slope PTP 0-4 min post-tetanus (% baseline)	**EPSP slope LTP 50-60 min post-tetanus (% baseline)**	**EPSP slope LTP 110-120 min post-tetanus (% baseline)**
Vehicle	145.2 ± 4.9	112.7 ± 2.8	110.3 ± 1.8
200 mM (*R,S*)-MCPG	145.7 ± 4.7	116.6 ± 1.3	109.8 ± 3.6

ANOVA results	$F < 1$	$F(1,10) = 1.22$; $p > 0.2$	$F < 1$

7.4.2.2 EPSP slope baseline changes

The slight baseline changes noted in the previous experiment were observed in all animals infused with MCPG. However, no significant differences in EPSP slope were found between vehicle and MCPG control pathways at any time point after tetanization (see analysis above), indicating that these

changes were transient and could not affect the level of LTP obtained. Comparison of the baseline period in figure 7.4.1A and B demonstrates that normalization of tetanized to control values smoothes out these baseline fluctuations since both pathways are affected in the same way by MCPG infusion (but see section 7.4.2.7).

7.4.2.3 EPSP slope I / O curves

Analysis of the EPSP slope I / O curves before and after tetanization revealed similar results to those described in experiment 7.3 (data not shown). The failure of MCPG to block LTP cannot be explained as an artifact of the test pulse stimulation intensities chosen.

7.4.2.4 Population spike LTP

For similar reasons to those described in experiment 7.3, population spike LTP was expressed as an absolute change in millivolts, rather than as a percentage increase. Figure 7.4.2A shows the population spike increase for both control and tetanized pathways, relative to the mean value over the 10 min prior to tetanization: MCPG had no effect on the induction of spike LTP. An ANOVA in which control and tetanized pathways were entered as sub groups revealed no group by pathway interaction 0-4 min, 50-60 min, or 110-120 min post tetanus (table 7.4.3).

Table 7.4.3 Two-pathway study: population spike potentiation.

	Increase in population spike amplitude 0-4 min post-tetanus (mV)		**Increase in population spike amplitude 50-60 min post-tetanus (mV)**		**Increase in population spike amplitude 110-120 min post-tetanus (mV)**	
	tetanized	**non-tetanized**	**tetanized**	**non-tetanized**	**tetanized**	**non-tetanized**
Vehicle	4.06 ± 0.68	0.06 ± 0.11	2.78 ± 0.49	0.13 ± 0.16	1.99 ± 0.30	0.32 ± 0.15
200 mM (*R,S*)-MCPG	4.80 ± 1.10	0.27 ± 0.14	3.02 ± 0.69	0.08 ± 0.10	2.43 ± 0.89	0.43 ± 0.19

ANOVA results	Drug group x pathway interaction NS [$F < 1$].	Drug group x pathway interaction NS [$F < 1$].	Drug group x pathway interaction NS [$F < 1$].

At each time point, the population spike change in a control pathway was subtracted from the corresponding change in the tetanized pathway; the normalized data are plotted in figure 7.4.2B. When analysed in this way, no significant differences were found in the amount of potentiation at any time point after tetanization (table 7.4.4)

Table 7.4.4 Two-pathway study: normalized population spike potentiation.

	Increase in population spike amplitude 0-4 min post-tetanus (mV)	**Increase in population spike amplitude 50-60 min post-tetanus (mV)**	**Increase in population spike amplitude 110-120 min post-tetanus (mV)**
Vehicle	4.0 ± 0.66	2.65 ± 0.46	1.67 ± 0.35
200 mM (*R,S*)- MCPG	4.53 ± 1.20	2.94 ± 0.71	2.0 ± 0.94
ANOVA results	$F < 1$	$F < 1$	$F < 1$

7.4.2.5 Baseline stimulation parameters

An ANOVA of absolute mean EPSP slope values over the 10 min before drug infusion, in which tetanized and control pathways were entered as sub-groups revealed no significant differences in initial slope values between drug groups, or between tetanized and control pathways (table 7.4.5). A similar ANOVA of mean absolute population spike amplitudes over the 10 min before drug infusion revealed no significant differences between groups or pathways (table 7.4.5). Analysis of test-pulse stimulation intensities revealed no significant differences between groups or between pathways (table 7.4.5).

Table 7.4.5 Two-pathway study: baseline parameters.

	Stimulation Intensity (μA)		**Pre-tetanus EPSP slope (mV/ms)**		**Pre-tetanus population spike amplitude (mV)**	
	tetanized	**non-tetanized**	**tetanized**	**non-tetanized**	**tetanized**	**non-tetanized**
Vehicle	330.0 ± 39.0	395.7 ± 39.9	3.18 ± 0.34	2.68 ± 0.17	2.70 ± 0.32	2.49 ± 0.21
200 mM MCPG	380.0 ± 58.3	420.0 ± 51.5	3.72 ± 0.31	3.24 ± 0.30	2.63 ± 0.55	2.48 ± 0.43

ANOVA results	**Effect of pathway:** $F(1,20) = 1.30$; $p > 0.2$ **Effect of drug group:** $F < 1$	**Effect of pathway:** $F(1,20) = 3.02$; $p > 0.05$ **Effect of drug group:** $F(1,20) = 3.64$; $p > 0.05$	**Effect of pathway:** $F < 1$ **Effect of drug group:** $F < 1$

7.4.2.6 Brain temperature

Infusion of MCPG caused a slight increase in brain temperature, relative to vehicle-infused controls, similar to that described in experiment 7.3. Figure 7.4.3 shows changes in temperature relative to the mean value over the 10 min prior to drug infusion. However, an ANOVA of the mean temperature increase over the 30 min after drug infusion, failed to reveal a significant group difference on this occasion (table 7.4.6).

No group differences in the mean temperature change obtained over the final 30 min of recording were found (table 7.4.6), but a slight increase in temperature over the course of the experiment was seen in both groups. Curiously, the difference in absolute pre-infusion baseline temperature reached significance (table 7.4.6). The reason for this difference is unknown, since an identical design of thermistor was used in each group, and MCPG-infused rats were interleaved with vehicle-infused rats over days. However, the difference is slight, and the result is based on a rather small sample of animals.

Table 7.4.6 Two-pathway study: brain temperature data

	Pre-infusion brain temp. (°C)	**Mean increase in brain temp. during 30 min after infusion (°C)**	**Mean increase in brain temp. during final 30 min of recording (°C)**
Vehicle	32.21 ± 0.57	0.12 ± 0.08	0.35 ± 0.07
200 mM (*R,S*)-MCPG	30.21 ± 0.26	0.36 ± 0.15	0.38 ± 0.21
ANOVA results	$F(1,10) = 7.69$; $p < 0.05$	$F(1,10) = 2.51$; $p > 0.1$	$F < 1$

7.4.2.7 Rats excluded from the above analysis

Seizures were induced in the right hippocampus of the first three animals prepared for bilateral recording (all vehicle-infused controls); these animals were excluded. Figure 7.4.4 shows the EPSP slope data from one such rat. The tetanized left-hand pathway shows a relatively stable baseline and normal, if modest, LTP. However, the non-tetanized right-hand pathway continues to rise throughout the experiment (figure 7.4.4A). The result when the tetanized pathway is normalized to the control is shown in figure 7.4.4B. Whilst the baseline change in this graph does not reach the criterion for rejection described below, it is clear that the apparent absence of LTP is due entirely to the seizure-

induced rise in the control pathway.

The problem was caused by the shape and position of the thermistor, which was positioned in the right hippocampus. It was found that an improved design, and a position roughly 2 mm posterior and 1 mm lateral to the right-hand recording electrode allowed the thermistor to be inserted without inducing a seizure. (After this change, only one further rat had to be excluded owing to a seizure in the right hippocampus.)

The bilateral design is intended to control for baseline changes, which might otherwise go unnoticed. However, if both pathways rise or fall to different extents during the baseline period before tetanization, then normalizing one to the other will be meaningless, since under these circumstances the control pathway does not provide any evidence about likely baseline changes in the tetanized pathway. Hence if normalization of the pathway to be tetanized to the control pathway resulted in a baseline which changed by more than 10 % over the hour prior to tetanization (i.e. a difference between the mean of the first 10 min and the last 10 min of greater than 10 %), then data from the animal concerned was excluded from further analysis.

This criterion led to the rejection of one vehicle and four MCPG-infused animals. The fact that four MCPG-infused animals had to be rejected on these grounds is potentially worrying; figure 7.4.5 shows an example. The control pathway was typically more depressed by MCPG infusion than the tetanized pathway. This may be partly because initial slope values in control pathways tended to be a little smaller in absolute terms than those in primary pathways (see table 7.4.5). However, it is possible that MCPG infusion interacted with damage caused by the nearby thermistor, resulting in a greater fall in baseline than that caused by drug infusion alone. (Note also that the baseline fall in the control pathway appears to have been fairly abruptly reversed following tetanization of the contralateral hemisphere. This phenomenon is considered in the discussion.)

Whatever the explanation, it could be argued that animals which showed the greatest baseline changes after drug infusion were those in which the infusion had been most successful. Since large changes in the baseline of each pathway increase the probability of divergence, it is possible that by applying a 10% change criterion to the final normalized result, those animals with the highest hippocampal concentrations of MCPG may be selected against. Nevertheless, it is meaningless to include data such as that shown in figure 7.3.4B, since the baseline rise in the normalized slope is merely an artefact.

In order to check that the selection criteria had not excluded those rats showing the greatest block of LTP, the primary pathway EPSP slope data from all animals studied were analysed, including those in which a seizure had been induced in the right hippocampus during electrode placement, but excluding one vehicle-infused rat in which an additional seizure was induced in the left hippocampus. Figure 7.4.6A shows the mean slope data for both MCPG and vehicle-infused groups, normalized to the pre-tetanus baseline. No significant group differences were observed 0-4 min, 50-60 min, or 110-120 min

post-tetanus (table 7.4.7).

However, as control pathway data from the original analysis suggest that the baseline change induced by MCPG infusion is transient (see figure 7.4.1A), it may be more appropriate to normalize all data to the pre-infusion baseline. Figure 7.4.6B shows the above results plotted in this way, calculated relative to the mean value over the 10 min before drug infusion. Both groups show almost identical levels of LTP (table 7.4.8).

The population spike increase was also equivalent in both groups whether normalized to the pre-tetanus baseline (table 7.4.9; figure 7.4.7A) or the pre-infusion baseline (table 7.4.10; figure 7.4.7B).

This analysis confirms that MCPG genuinely fails to block LTP under the current circumstances, and this failure cannot be an artifact of the criteria used to reject data from the main analysis.

Table 7.4.7 Two-pathway study: EPSP slope LTP data from tetanized pathways of all rats tested. Values are normalized to the **pre-tetanus** baseline.

	EPSP slope PTP 0-4 min post-tetanus (% baseline)	**EPSP slope LTP 50-60 min post-tetanus (% baseline)**	**EPSP slope LTP 110-120 min post-tetanus (% baseline)**
Vehicle	142.0 ± 4.2	115.9 ± 1.9	111.9 ± 1.7
200 mM (*R,S*)-MCPG	144.7 ± 4.7	121.1 ± 3.1	115.5 ± 3.0
ANOVA results	$F < 1$	$F(1,18) = 2.02$; $p > 0.1$	$F(1,18) = 1.24$; $p > 0.2$

Table 7.4.8 Two-pathway study: EPSP slope LTP data from tetanized pathways of all rats tested. Values are normalized to the **pre-infusion** baseline.

	EPSP slope PTP 0-4 min post-tetanus (% baseline)	**EPSP slope LTP 50-60 min post-tetanus (% baseline)**	**EPSP slope LTP 110-120 min post-tetanus (% baseline)**
Vehicle	144.9 ± 5.5	118.1 ± 2.8	114.0 ± 2.5
200 mM (*R,S*)-MCPG	141.4 ± 6.1	118.0 ± 3.4	112.7 ± 3.6
ANOVA results	$F < 1$	$F < 1$	$F < 1$

Table 7.4.9 Two-pathway study: population spike LTP in tetanized pathways of all rats tested. Values are normalized to the **pre-tetanus** baseline.

	Increase in population spike amplitude 0-4 min post-tetanus (mV)	**Increase in population spike amplitude 50-60 min post-tetanus (mV)**	**Increase in population spike amplitude 110-120 min post-tetanus (mV)**
Vehicle	4.48 ± 0.49	2.76 ± 0.39	1.81 ± 0.33
200 mM (*R,S*)-MCPG	4.57 ± 0.70	2.88 ± 0.45	2.37 ± 0.51
ANOVA results	$F < 1$	$F < 1$	$F < 1$

Table 7.4.10 Two-pathway study: population spike LTP data from tetanized pathways of all rats tested. Values are normalized to the **pre-infusion** baseline.

	Increase in population spike amplitude 0-4 min post-tetanus (mV)	**Increase in population spike amplitude 50-60 min post-tetanus (mV)**	**Increase in population spike amplitude 110-120 min post-tetanus (mV)**
Vehicle	4.41 ± 0.50	2.69 ± 0.36	0.33 ± 1.74
200 mM (*R,S*)-MCPG	3.82 ± 0.70	2.13 ± 0.44	1.62 ± 0.50
ANOVA results	$F < 1$	$F(1,18) = 1.01$; $p > 0.3$	$F < 1$

Despite some of the problems described above, the bilateral set-up worked well much of the time. Figure 7.4.8A shows both tetanized and control pathways from one such experiment (200 mM (*R,S*)-MCPG infusion). Fluctuations in EPSP slope are broadly paralleled by temperature changes (figure 7.4.8B) suggesting a possible explanation for these irregularities. When the tetanized pathway is normalized to the control pathway [(tetanized / control) x 100, at each time point; figure 7.4.8C], gradual changes are smoothed out.

7.4.3 Discussion

These results confirm the original finding that MCPG is ineffective in blocking LTP. Furthermore, the slight baseline changes caused by MCPG appear to be transient, and hence unlikely to affect a measure of LTP recorded 1-2 hr later. However, the baseline fall in the control pathway following MCPG infusion appears to reverse quite abruptly after contralateral tetanization (see figures 7.4.1A, 7.2.5 and 7.2.8), suggesting that control and tetanized pathways may not be strictly independent. A similar effect was observed in rats infused with buffered saline. One complication with the use of the contralateral perforant path–dentate gyrus projection as a control pathway is that each hippocampus receives a commissural projection from the contralateral dentate gyrus. Hence, the possibility of heterosynaptic potentiation of the control pathway after contralateral tetanization may provide an explanation for the slight rise in control pathways seen after tetanization. However, whilst a heterosynaptic depression of the contralateral response has been found following tetanization of the ipsilateral perforant path, heterosynaptic potentiation of the ipsilateral projection following contralateral potentiation has not been observed (Levy and Steward, 1979; Krug et al., 1985). Nevertheless, even if the two pathways used in the current experiment were not strictly independent, the analysis of all tetanized pathways presented in figure 7.4.5 demonstrates very clearly that MCPG has no effect on LTP under these circumstances.

The reason for the apparent slight reduction in LTP after 200 mM (*R,S*)-MCPG infusion in experiment 7.3 is unknown. However, this reduction was not significant, and did not occur in the present experiment; the original result might simply have been the result of chance variation in the level of LTP.

A further potential problem concerns the unknown concentration of MCPG reaching the hippocampus after i.c.v. infusion. The failure to find an effect of a drug on LTP without any positive control constitutes a very weak result. Unlike D-AP5 which causes a characteristic fall in population spike amplitude and, of course, blocks LTP, MCPG does not cause any obvious indication of its presence, except for a slight fall in EPSP slope baseline. Furthermore, MCPG is not readily detected, at least in our hands, by HPLC. In order to overcome these problems, the experiment described below was carried out in order to test the efficacy of MCPG infusion in blocking the effects of an mGluR agonist.

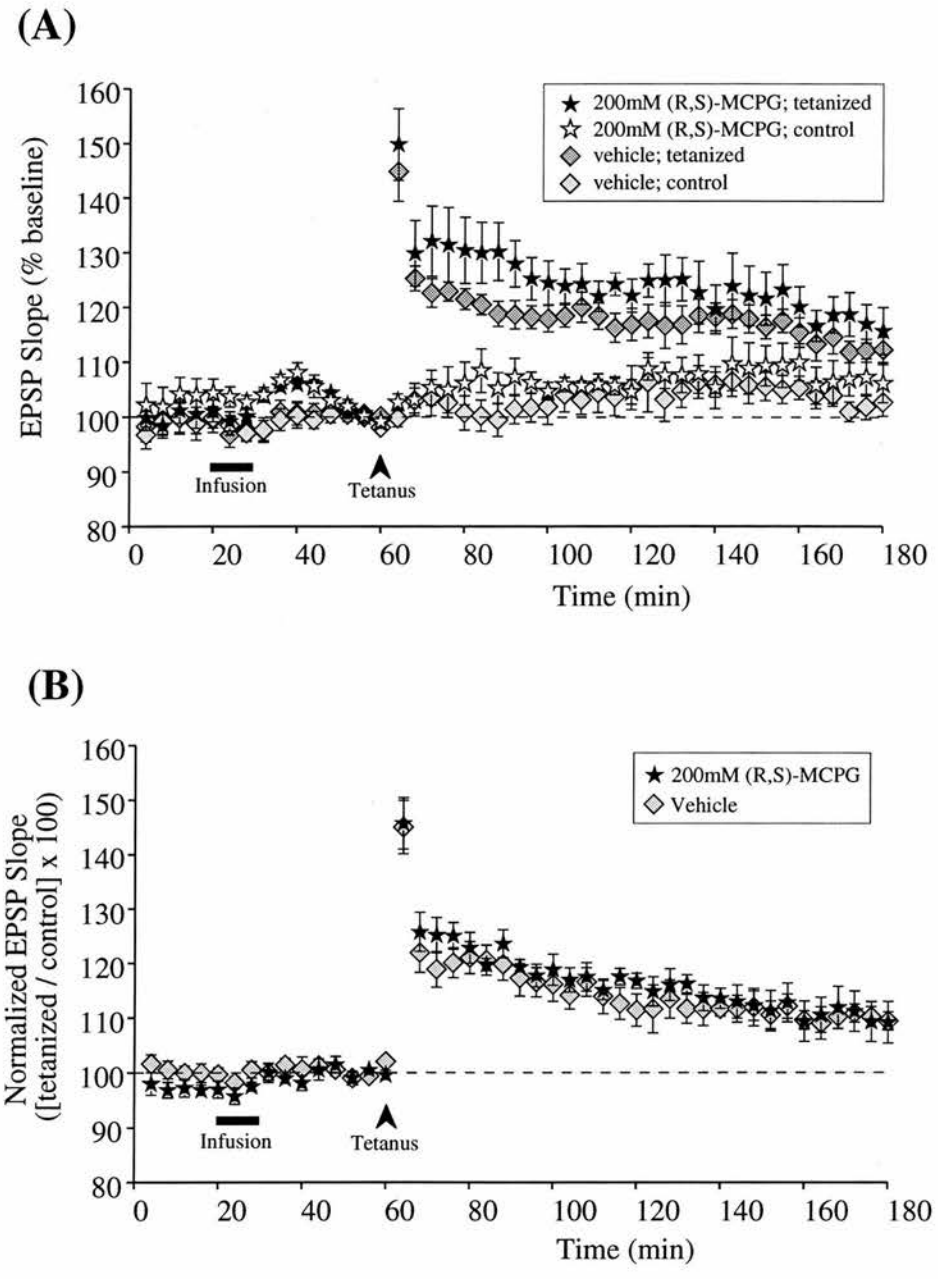


Fig. 7.4.1

(A) Infusion of 200 mM (R,S)-MCPG (n = 5) failed to block EPSP slope LTP relative to vehicle-infused controls (n = 7). Tetanized pathways in each group showed equivalent LTP; control pathways did not diverge during the course of the experiment, indicating that MCPG infusion did not result in a chronic baseline fall.
(B) Tetanized pathways normalized to control pathways at each time point. Normalized LTP was identical in both groups.

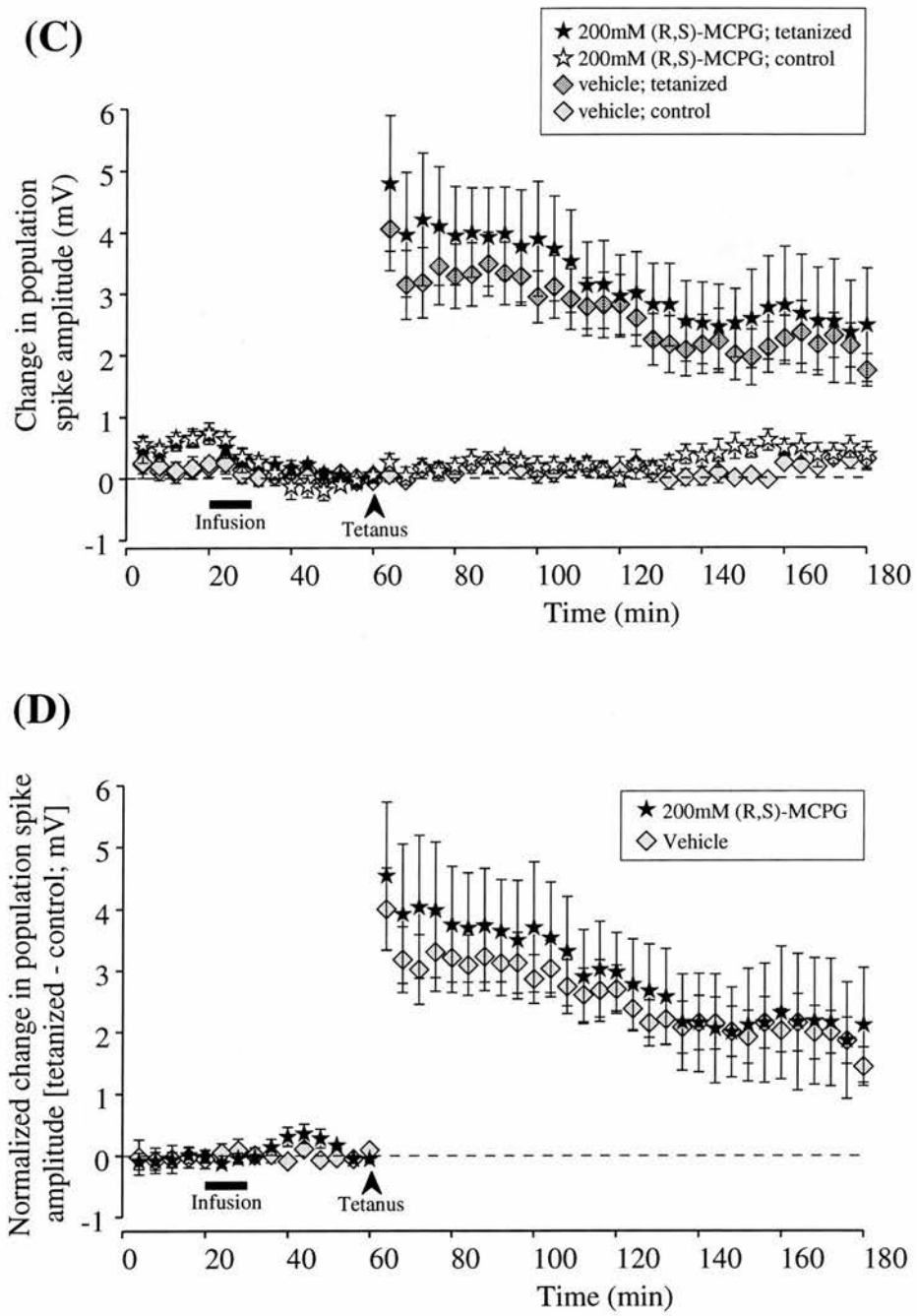


Fig. 7.4.2

(C) Equivalent increases in population spike amplitude were seen in rats infused with 200 mM (R,S)-MCPG (n = 5) or vehicle (n = 7). Non-tetanized pathways likewise did not differ. (D) Tetanized pathways normalized to control pathways at each time point. Equivalent LTP was seen in the two groups.

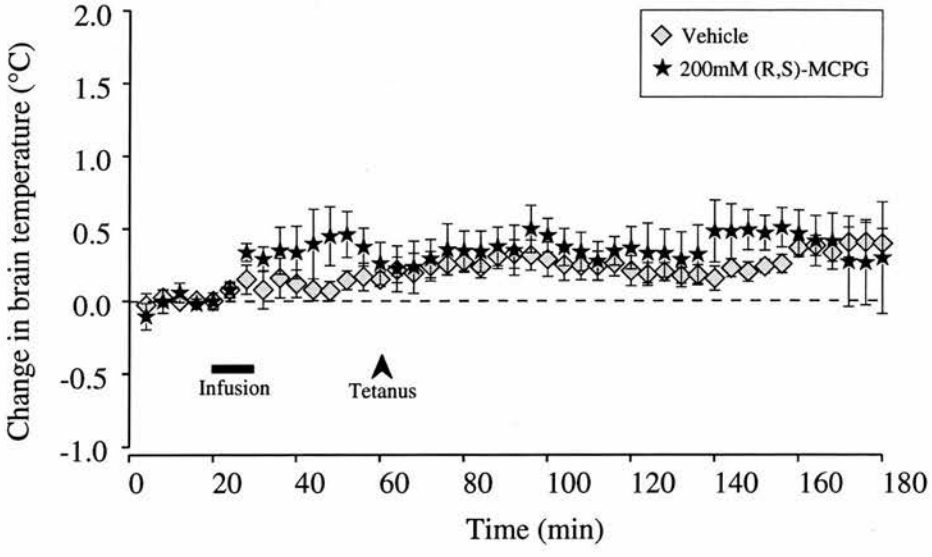


Fig 7.4.3

Mean change in brain temperature during bilateral stimulation and recording. Infusion of 200 mM (R,S)-MCPG caused a slight increase in brain temperature relative to vehicle-infused controls.

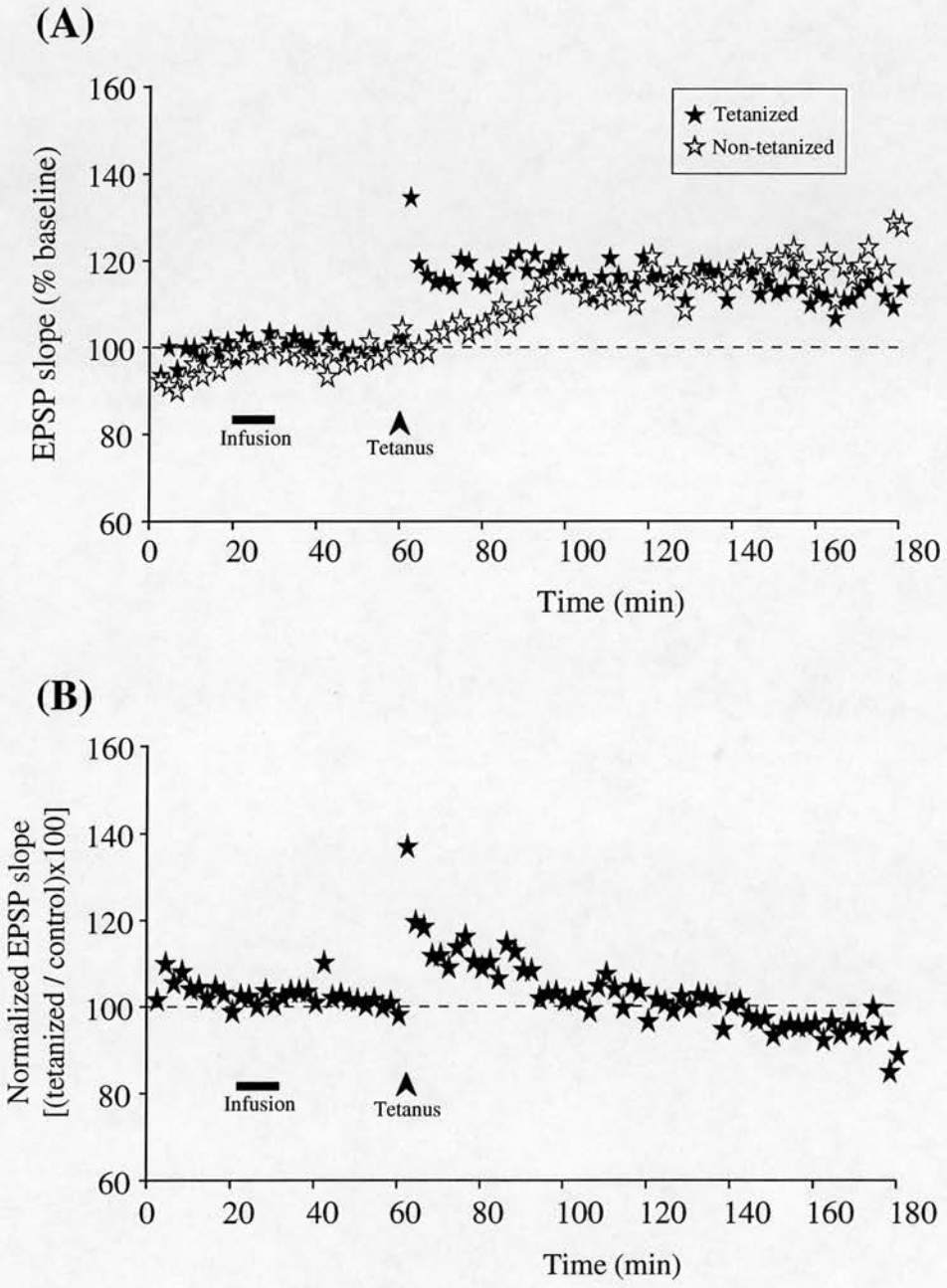


Fig. 7.4.4

(A) EPSP slope data for both the tetanized and the non-tetanized pathway in an individual rat infused with PBS. Data from this animal were not included in the main analysis owing to the fact that a seizure was induced in the non-tetanized pathway during positioning of the recording electrode and thermistor. Note the subsequent chronic rise in this pathway. (B) The apparent absence of LTP in the normalized slope data is an artifact of the chronic rise in the control pathway.

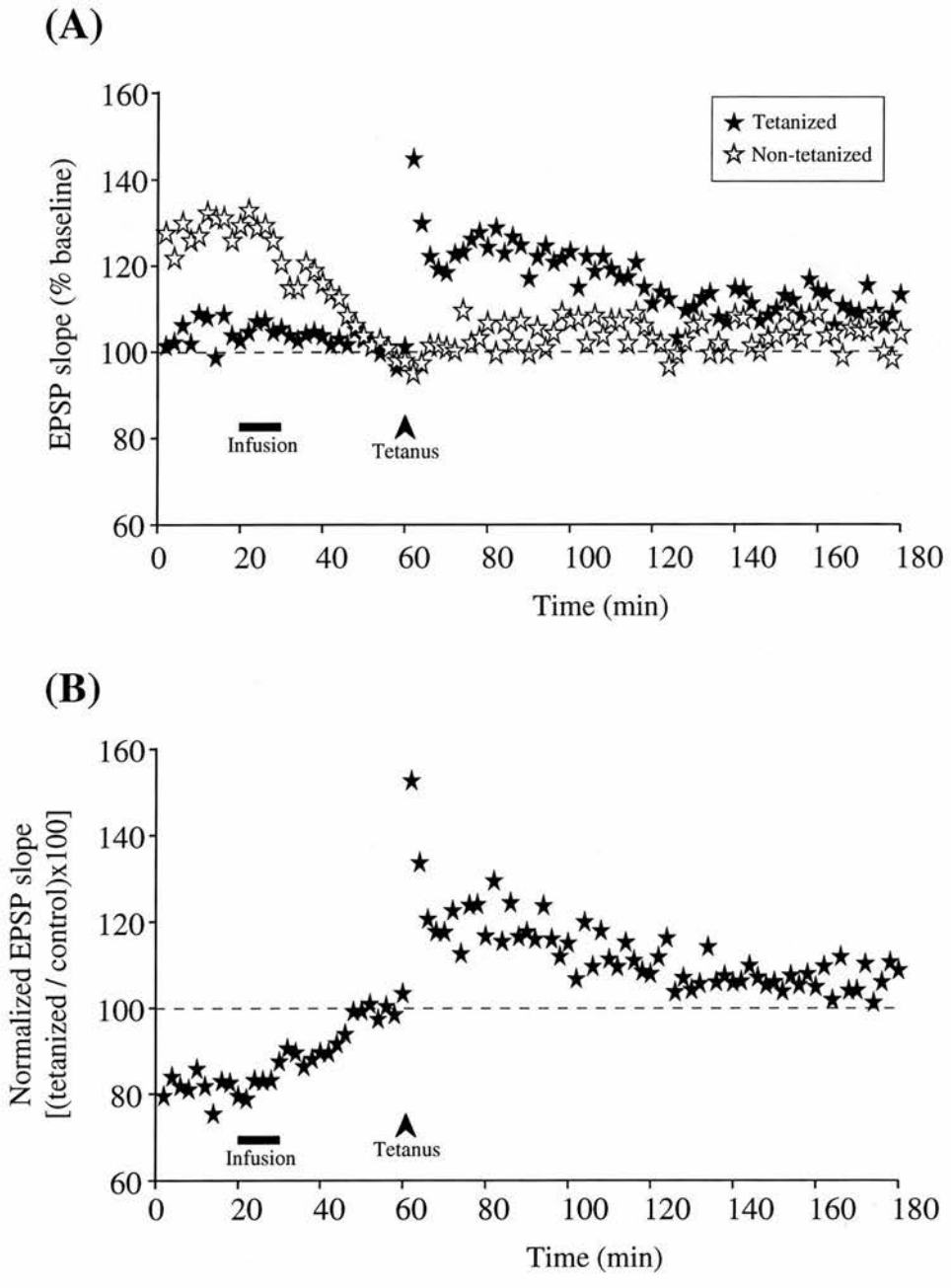


Fig. 7.4.5

(A) EPSP slope data for both the tetanized and the non-tetanized pathway in an individual rat infused with 200 mM (R,S)-MCPG. Data from this animal were not included in the main analysis owing to the fact that the normalized baseline shown in (B) changed by more than 10% over the 60 min prior to tetanization.

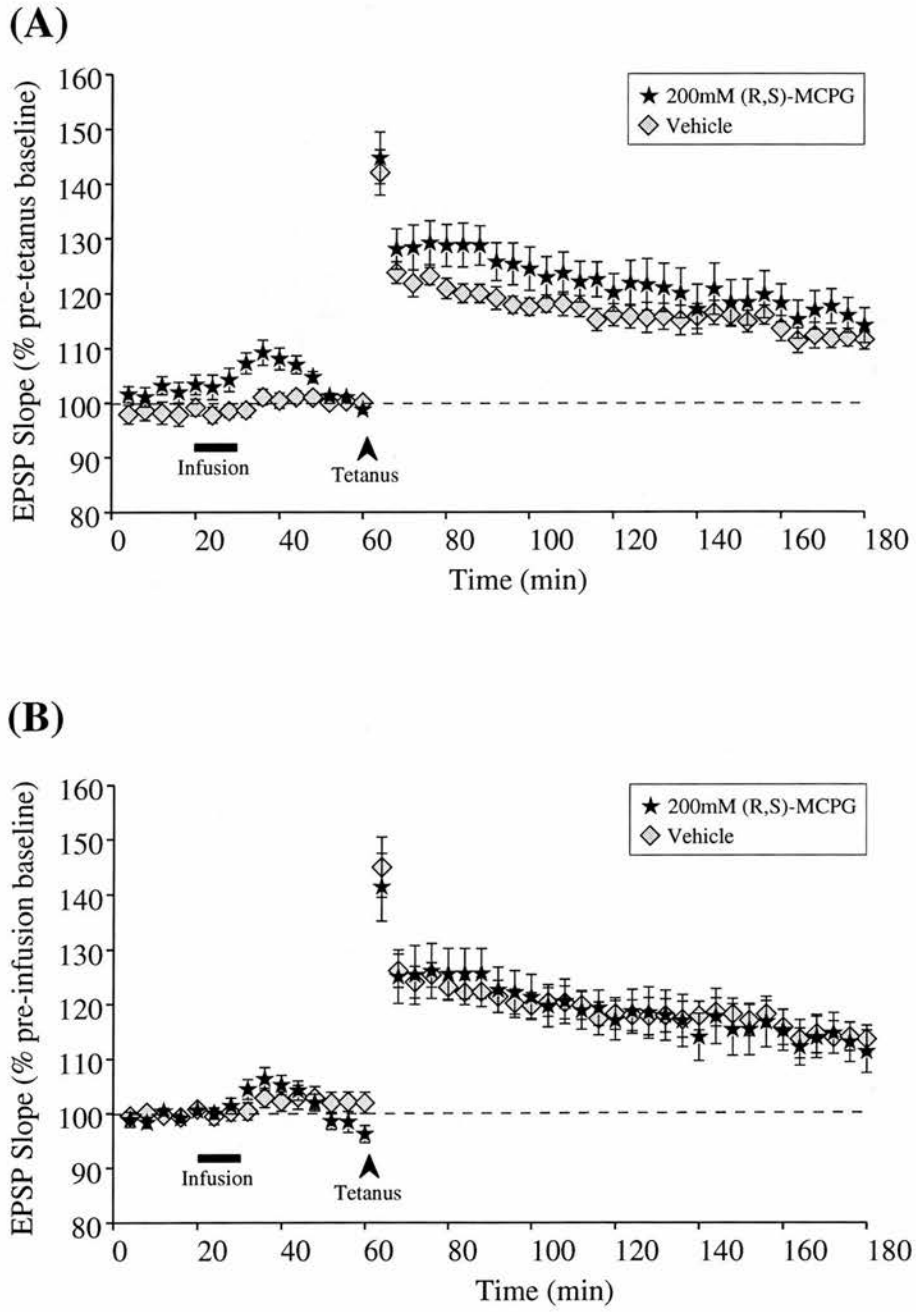


Fig 7.4.6

EPSP slope LTP data from the tetanized pathways of all rats tested, including those rejected from the main analysis. (A) Data normalized to the pre-tetanus baseline. (B) Data normalized to the pre-infusion. baseline. The failure of MCPG to block LTP cannot be an artifact of the criteria used to reject failed experiments.

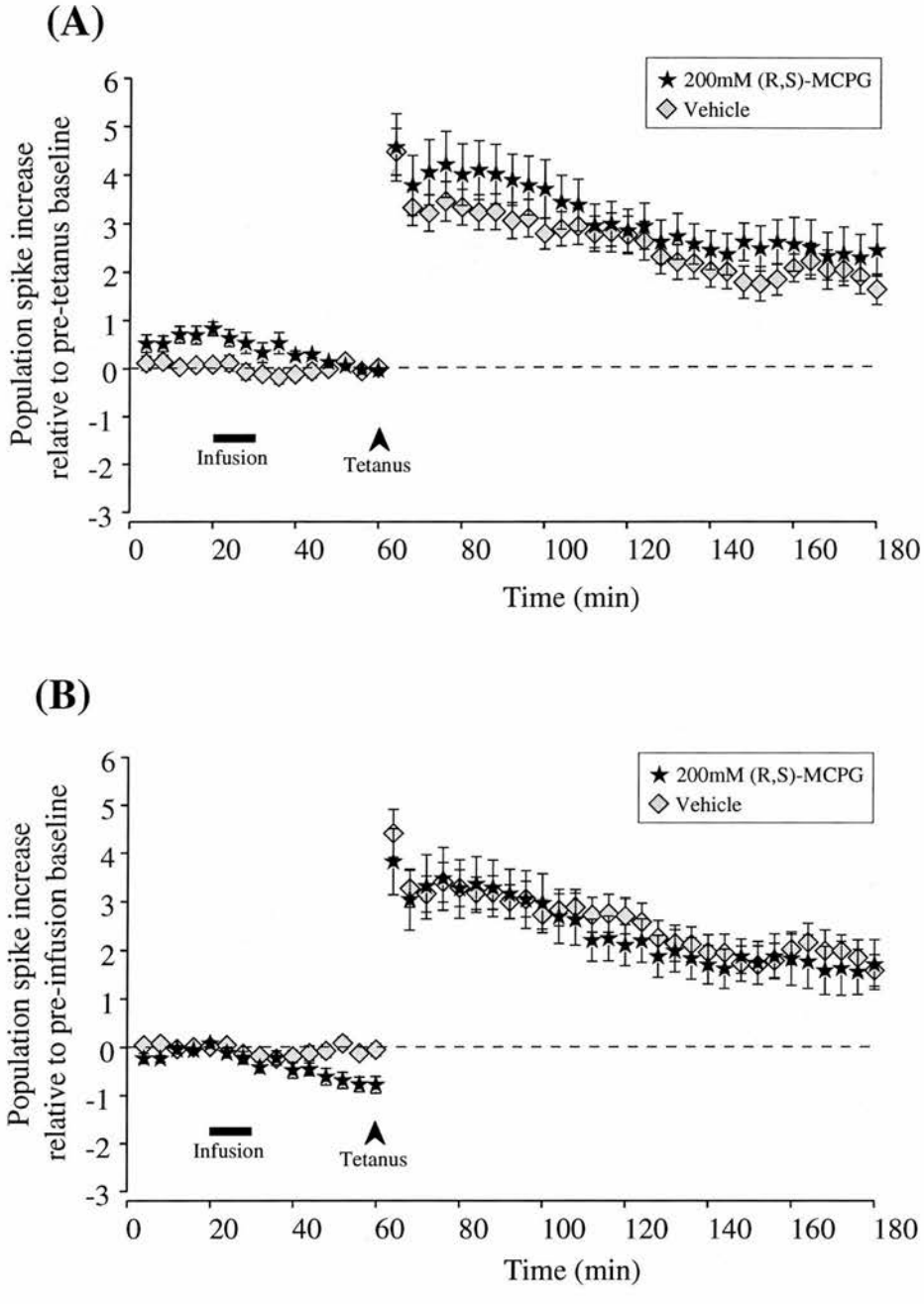


Fig 7.4.7

Population spike LTP data from the tetanized pathways of all rats tested, including those rejected from the main analysis. (A) Data normalized to the pre-tetanus baseline. (B) Data normalized to the pre-infusion basline. The failure of MCPG to block LTP cannot be an artifact of the criteria used to reject failed experiments.

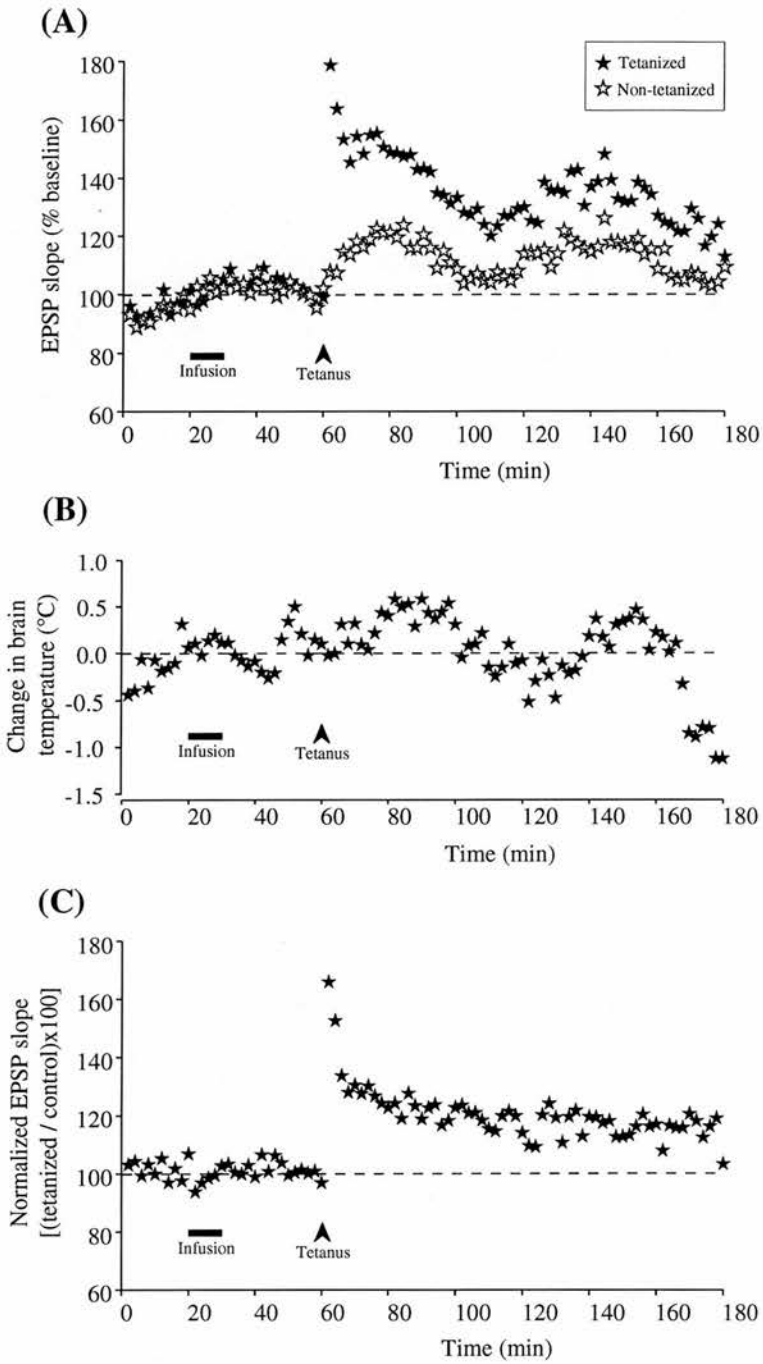


Fig. 7.4.8

(A) EPSP slope data for both the tetanized and the non-tetanized pathway in an individual rat infused with 200 mM (R,S)-MCPG. Data from this animal were included in the main analysis. (B) Changes in brain temperature throughout the experiment. Note that the fluctuations are broadly parallelled by changes in EPSP slope in the figure above. (C) Normalization of tetanized to control values smoothes out fluctuations in the data.

7.5 Does MCPG antagonize the electrophysiological effects of the mGluR agonist (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) *in vivo*?

7.5.1 Introduction

In order to check whether the i.c.v. infusion of 200 mM (*R*,*S*)-MCPG in the previous experiments was sufficient to cause a functional blockade of mGluRs in the dentate gyrus, the ability of MCPG to inhibit the actions of the broad spectrum mGluR agonist (1*S*,3*R*)-ACPD was tested. However, it was first necessary to establish the existence of a suitable ACPD-mediated response. Application of (1*S*,3*R*)-ACPD has been reported to have a wide range of physiological effects in a number of different experimental preparations (see chapter 3), although few studies have addressed the effects of ACPD in the dentate gyrus *in vivo*.

Manahan-Vaughan and Reymann (1995b) reported that (1*S*,3*R*)-ACPD induced a slow-onset potentiation in dentate field EPSPs *in vivo*. However, others have found only a transient potentiation, followed by depression at higher doses (Davis and Laroche, 1996). Brain temperature was not monitored in these studies, although the slow-onset potentiation of population spike amplitude in addition to EPSP slope obtained by Manahan-Vaughan and Reymann (1995b) would suggest that the effect is not merely a temperature artifact. (Population spike amplitude is usually observed to fall after a temperature increase; see chapter 2.6.2). However, application of ACPD has been found to cause an increase of almost 1° C in the temperature recorded at the brain surface (R. C. Heath, unpublished observations). This temperature rise persisted for the duration of the experiment (1.5 hr) and may be an underestimate of the true increase in hippocampal temperature. The highest dose of ACPD tested caused a transient EPSP slope potentiation of roughly 20 %, which fell almost to baseline levels within 1.5 hr after the start of infusion. This dose of ACPD did not cause a potentiation of population spike amplitude; in fact a fall in population spike was sometimes observed. As discussed below, a temperature rise of 1° C would be sufficient to account for the observed increase in EPSP slope. The dissociation between EPSP slope and population spike is also consistent with the possibility of a temperature artifact.

However, as mentioned above, a potentiation of population spike amplitude was reported by Manahan-Vaughan and Reymann (1995a). Hence, even if the increase in EPSP slope after ACPD infusion is purely a temperature artifact, the population spike increase may be due to different mechanisms, such as an increase in neuronal excitability, or a reduction in inhibitory tone (see chapter 3.9 & 3.11).

Studies *in vivo* have concentrated on the role of mGluR activation on evoked field potentials. However, application of ACPD has been reported to cause an increase in the spontaneous

synchronous activity of CA1 pyramidal cells, attributed to mGluR-driven network oscillations of inhibitory interneurons (Whittington et al., 1995; Boddeke et al., 1997). The change in spontaneous dentate activity provides a further possible assay for mGluR activation, and its blockade by MCPG.

The aim of the following experiments was firstly to characterize the effects of i.c.v. infusion of (1*S*,3*R*)-ACPD on dentate EPSP slope, population spike amplitude, spontaneous activity, and temperature. The ability of MCPG to block these effects was then assessed.

7.5.2 Methods

The recording set-up was identical to that described in chapter 4. A range of doses of (1*S*,3*R*)-ACPD were tested: 25 mM, 4 mM, 2 mM and 1 mM. Brain temperature was monitored in the 1 mM and 2 mM groups only. In these animals, a thermistor was placed in the dentate gyrus contralateral to the stimulation and recording electrodes. Spontaneous hippocampal activity was monitored by polygraph, and recorded for off-line analysis using an FM reel-to-reel tape recorder (Racal-Thermionic Ltd.). Following a 20 min baseline period, rats received a bilateral i.c.v. infusion of 10µl (5µl per ventricle) of either phosphate buffered saline or 200 mM (*R*,*S*)-MCPG over 10 minutes. Thirty minutes later, a 10 µl infusion of 1 mM (1*S*,3*R*)-ACPD was given, again over 10 min. In all cases except the 25 mM group, a second infusion of (1*S*,3*R*)-ACPD was given 2 hr after the start of the first infusion of this drug in order to investigate the "wash-out" of (*R*,*S*)-MCPG. Recording was continued for a further 50 min after the end of this infusion.

7.5.3 Results

7.5.3.1 25 mM (1*S*,3*R*)-ACPD

In an initial pilot study, two rats were infused with PBS followed by ACPD; two more were first infused with 200 mM (*R*,*S*)-MCPG. Only one infusion of 25 mM (1*S*,3*R*)-ACPD was given to each animal. The 25 mM dose was chosen to match that used in a previous study carried out in this laboratory (R. C. Heath, unpublished observations). ACPD infusion caused a marked rise in EPSP slope of almost 20 %, which fell back to baseline within about 30 min of the end of infusion (data not shown). This transient increase was not blocked by MCPG. ACPD also caused a slight rise in population spike that was not blocked by MCPG (data not shown). Brain temperature and spontaneous dentate activity were not monitored in these animals.

7.5.3.2 4mM (1*S*,3*R*)-ACPD

The possibility that MCPG might block the rise in EPSP slope induced by a lower concentration of ACPD was tested by reducing the dose from 25 mM to 4 mM. Spontaneous dentate activity was also recorded as an alternative index of mGluR activation. On this occasion, a second infusion of ACPD was delivered 2 hr after the start of the first. Four animals were infused with 200 mM (*R*,*S*)-MCPG before ACPD; another four were initially infused with PBS.

Changes in EPSP slope

Figure 7.5.1A shows the EPSP slope data throughout the experiment, normalized to the 10 min immediately before the infusion of PBS / MCPG. In figure 7.5.1B, data are shown over the range starting 20 min before, and ending 40 min after, the start of each ACPD infusion. Data are normalized to the mean value over the 10 min before each individual infusion; i.e. each portion of the data is normalized to its own, separate baseline. This eliminates variation due to the MCPG-induced baseline fall, as well as chronic baseline changes that may have occurred over the course of an experiment. Both infusions of ACPD caused a transient increase in the EPSP slope, the increase being slightly more pronounced after the second infusion. The maximum increase in slope was similar to that obtained after 25 mM (1*S*,3*R*)-ACPD infusion, although the onset of the rise was slightly slower, and the fall back towards baseline values was a little more rapid. As before, the increase in EPSP slope was not blocked by prior infusion of MCPG.

Figure 7.5.2 shows the change in population spike throughout the experiment, analysed in the same way as the EPSP slope data. Despite the variability of the data, and the severe fall in baseline caused by MCPG infusion, it appears that ACPD caused a slight transient increase in population spike amplitude of up to 1 mV, which was not prevented by the prior infusion of MCPG.

Changes in spontaneous dentate EEG activity

Spontaneous EEG activity was recorded via the same electrode used to monitor field potentials. Recording was carried out over a period starting 2 min before ACPD infusion and finishing 10 min after the end of infusion. For an example of this form of recording, see figure 7.5.3A (The data are taken from the 1 mM ACPD experiment detailed below). The figure is a representative polygraph trace recorded during and after the infusion of ACPD in a rat previously infused with buffered saline. The large downward deflections represent positive-going EPSPs, spaced at 20 s intervals; upward deflections reflect the subsequent hyperpolarization. The continuous low amplitude noise recorded between EPSPs represents spontaneous hippocampal neuronal activity in the absence of afferent

stimulation. ACPD reliably caused an increase in the amplitude of this spontaneous activity. An equivalent polygraph trace from a rat previously infused with 200 mM (*R,S*)-MCPG is shown in figure 7.5.3B. On this occasion, the increase in noise was completely blocked.

The amplitude of spontaneous activity was measured directly from the trace, samples being taken every 2 min. The mean change in amplitude of spontaneous activity is presented in figure 7.5.4. In part A, the change in spontaneous noise is plotted relative to the 2 min prior to each ACPD infusion. Spontaneous activity was not routinely recorded during infusion of PBS or MCPG since pilot studies indicated that no changes in spontaneous activity resulted from the infusion of these drugs (PBS: n = 3; 200 mM (*R,S*)-MCPG: n = 5; data not shown). The first infusion of ACPD caused a marked rise in spontaneous activity, which was completely blocked by the prior infusion of 200 mM (*R,S*)-MCPG. A partial blockade of the increase in spontaneous activity induced by the second ACPD infusion 2½ hr later was also observed, indicating that washout of MCPG was incomplete. Two of the rats initially infused with PBS showed evidence of epileptiform activity during the second ACPD infusion; such activity was never observed in rats initially infused with MCPG (data not shown).

The increase in spontaneous activity following the first ACPD infusion is plotted in expanded form in figure 7.5.4B. The effects of ACPD infusion appear to be biphasic. MCPG infusion unmasks a depression induced by ACPD, but blocks the rise in spontaneous activity. These effects may reflect the different affinities of MCPG and ACPD for individual mGluR subtypes (see discussion). An ANOVA of the data presented in figure 7.5.4B, in which time points were entered as a within subject factor, revealed a significant overall group difference between MCPG and PBS treated rats [$F(1,6) = 12.01$; $p < 0.05$], together with a significant group by time point interaction [$F(10,60) = 2.58$; $p < 0.05$]. Significant group differences at individual time points are indicated by asterisks. This analysis demonstrates that MCPG significantly antagonizes the ACPD-induced rise in spontaneous dentate activity. The mean peak increase in spontaneous activity following each infusion of ACPD is summarized in table 7.5.1. No significant group differences were found in absolute pre-infusion levels of spontaneous activity measured over the 2 min prior to drug infusion (table 7.5.2).

Table 7.5.1 Maximum mean increase in the amplitude of spontaneous dentate activity induced by of 4 mM (1*S*,3*R*)-ACPD, in the presence or absence of 200 mM (*R,S*)-MCPG.

	Maximum mean increase in amplitude of spontaneous activity (mV)	
	1st ACPD infusion	**2nd ACPD infusion**
PBS + 4 mM ACPD	0.81 ± 0.44	1.60 ± 0.89
200 mM MCPG + 4mM ACPD	0.14 ± 0.25	0.48 ± 0.02

Table 7.5.2 Initial amplitude of spontaneous dentate activity over the 2 min prior to ACPD infusion.

	Amplitude of spontaneous activity (mV)	
	before 1st ACPD infusion	**before 2nd ACPD infusion**
PBS + 1 mM ACPD	1.15 ± 0.11	1.24 ± 0.10
200 mM MCPG + 1mM ACPD	1.30 ± 0.09	1.36 ± 0.07

ANOVA result Difference between drug groups:-	$F < 1$
Difference between first and second ACPD infusion:-	$F < 1$

7.5.3.3 2 mM (1*S*,3*R*)-ACPD

Despite the finding that MCPG can block the rise in spontaneous dentate activity induced by 4 mM ACPD, it was decided to reduce the dose of ACPD in order to investigate whether MCPG might also reduce the rise in EPSP slope under these circumstances. However, it was also decided to monitor brain temperature in order to determine to what extent the rise in slope could be accounted for by an increase in temperature. A second reason for lowering the dose concerned the fact that epileptiform discharges were sometimes observed during the second infusion of 4 mM ACPD, suggesting that this concentration may be excessively high.

Before starting the 1 mM experiment, however, two rats were infused with 2 mM ACPD; one received an initial infusion of PBS, the other received 200 mM (*R*,*S*)-MCPG. Although essentially pilot data, the findings from these animals are included since they provide clear individual examples of the parallel rise in brain temperature and EPSP slope following ACPD infusion.

Changes in EPSP slope

Figure 7.5.5 shows the change in EPSP slope analysed in the same way as in figure 7.5.1. Infusion of ACPD induced an EPSP slope increase that was only slightly smaller that that induced by infusion of 4 mM ACPD. As before, this increase was not blocked by prior infusion of MCPG. Owing to the baseline variability of the data, it was impossible to judge whether population spike amplitude was also increased (data not shown). Figure 7.5.6 shows the increase in brain temperature induced by ACPD infusion, analysed in the same way as the EPSP slope data, i.e. normalized to the baseline immediately prior to each infusion of ACPD. ACPD infusion caused a marked temperature rise of

approximately 1 °C in controls, a rise that was little affected by prior infusion of MCPG. It is notable that the time course of this transient temperature rise was closely paralleled by the rise in slope plotted in figure 7.5.5.

Changes in spontaneous EEG activity

The change in spontaneous activity in the two rats infused with 2 mM ACPD is plotted in figure 7.5.7, analysed in the same way as the data shown in figure 7.5.4. However, the ACPD-induced increase in noise is rather modest in the PBS-infused animal, and the pre-infusion baseline noise is unstable in the MCPG-infused rat. Hence, few conclusions can be drawn about the effect of MCPG on ACPD-induced noise from these individual examples.

7.5.3.4 1 mM (1*S*,3*R*)-ACPD

In the final experiment, rats were infused with 1 mM ACPD. The experimental set-up was identical to that described above. However, at the end of the experiment, 7 rats from each group infused with 1mM (1*S*,3*R*)-ACPD were passively warmed, and regression lines were calculated relating EPSP slope to brain temperature.

Changes in spontaneous EEG activity

Figure 7.5.3A shows a representative polygraph trace recorded during and after the first 1 mM ACPD infusion in a rat previously infused with buffered saline. An equivalent polygraph trace recorded from a rat previously infused with 200 mM MCPG is shown in figure 7.5.3B. On this occasion, the increase in noise was completely blocked.

Figure 7.5.8A shows the mean increase in amplitude of spontaneous activity in each group analysed in the same way as in figure 7.5.4A. As before, an expanded figure of the rise in spontaneous activity following the first ACPD infusion is shown in part B. The maximum increase in spontaneous activity from baseline values is shown in table 7.5.3. An ANOVA of all data points from 60-80 min after the start of the experiment, in which time was entered as a within subject factor, revealed a significant group difference [$F(1,14) = 7.39$; $p < 0.05$], and a significant group by time point interaction [$F(10,140) = 2.37$; $p < 0.05$]. Subsequent analysis of simple effects revealed that spontaneous activity levels were significantly higher in the PBS / ACPD group than in the MCPG / ACPD group for a period 6 min after the start of ACPD infusion until 2 min after the end of infusion (see graph legend). However, inspection of figure 7.5.8B suggests that prior infusion of MCPG unmasked a slight initial

depression in spontaneous activity induced by ACPD.

The second infusion of 1 mM ACPD resulted in a slightly greater, but still transient, increase in spontaneous activity which was only partially blocked by prior infusion of MCPG 180 min earlier, suggesting incomplete washout of the drug (figure 7.5.8A; table 7.5.3).

Table 7.5.3 Maximum mean increase in the amplitude of spontaneous dentate activity induced by of 1 mM (1*S*,3*R*)-ACPD, in the presence or absence of 200 mM (*R*,*S*)-MCPG.

	Maximum mean increase in amplitude of spontaneous activity (mV)	
	1st ACPD infusion	**2nd ACPD infusion**
PBS + 1 mM ACPD	0.96 ± 0.22	1.11 ± 0.18
200 mM MCPG + 1 mM ACPD	0.18 ± 0.29	0.54 ± 0.22

In order to check for chronic effects of ACPD application that might have carried over from the first to the second infusion, an ANOVA of the baseline pre-infusion spontaneous activity levels was carried out. Values prior to the first and second ACPD infusions were entered as within subject factors. An ANOVA revealed neither an overall group difference between MCPG and PBS-treated rats, nor an overall difference between baselines prior to the first ACPD infusion and prior to the second (table 7.5.4). A separate ANOVA of baseline spontaneous activity in the PBS pre-treated group alone also failed to reveal a significant difference between these two baseline periods [$F < 1$], confirming that the effects of 1 mM ACPD infusion are indeed transient.

Table 7.5.4 Initial amplitude of spontaneous dentate activity over the 2 min prior to ACPD infusion.

	Amplitude of spontaneous activity (mV)	
	before 1st ACPD infusion	**before 2nd ACPD infusion**
PBS + 1 mM ACPD	1.15 ± 0.11	1.24 ± 0.10
200 mM MCPG + 1mM ACPD	1.30 ± 0.09	1.36 ± 0.07

ANOVA results Difference between drug groups:-	$F(1,14) = 1.39$; $p > 0.2$
Difference between first and second ACPD infusion:-	$F(1,14) = 1.34$; $p > 0.2$

Spectral analysis of spontaneous activity

A preliminary spectral analysis of the polygraph record before and after 1 mM ACPD infusion indicates that the increase in amplitude of spontaneous dentate activity consists of a selective increase in frequencies between 20 and 50 Hz (data not shown). The significance of this result will be discussed later.

Changes in EPSP slope

Infusion of ACPD was again associated with a transient rise in EPSP slope; an effect not blocked by MCPG (figure 7.5.9; data analysed as in figure 7.5.1). The increase in EPSP slope following each infusion of 1 mM ACPD was measured as the percentage change in mean value from the baseline recorded over the 10 min immediately prior to that infusion, to the mean value over the 10 min following the end of infusion (table 7.5.4). An ANOVA in which the first and second infusions of ACPD were entered as within subject factors revealed no overall difference between the increase in slope following the first ACPD infusion compared to that following the second infusion; animals previously infused with MCPG showed equivalent increases to rats previously infused with PBS (table 7.5.4). However, the increase in slope caused by ACPD infusion was transient and always returned to baseline within 20 min of the end of infusion.

Table 7.5.4 Mean increase in EPSP slope averaged over the 10 min following each infusion of ACPD.

	Mean percentage increase in EPSP slope (% baseline)	
	after 1st ACPD infusion	**after 2nd ACPD infusion**
PBS + 1 mM ACPD	5.23 ± 2.12	9.00 ± 1.54
200 mM MCPG + 1 mM ACPD	5.39 ± 1.75	6.02 ± 1.56

ANOVA result Overall difference between PBS and MCPG groups:	$F < 1$
Overall difference between first and second infusion of ACPD:	$F(1,14) = 2.10$; $p > 0.1$
Drug group x infusion interaction:	$F(1,14) = 1.08$; $p > 0.3$

Changes in population spike amplitude

Figure 7.5.10 shows the change in population spike throughout the experiment, analysed in the same way as the EPSP slope data. Despite the variability of the data, and the severe fall in baseline caused by MCPG infusion, it appears that ACPD caused a very slight transient increase in population spike amplitude, which was not blocked by prior infusion of MCPG. The increase was most evident after the second ACPD infusion, possibly owing to the stable pre-infusion baseline at this point. Population spike changes following the second ACPD infusion are considered in greater detail in connection with the increase in brain temperature associated with ACPD infusion (see below).

Changes in brain temperature

The increase in EPSP slope described above was typically accompanied by a slight transient rise in brain temperature whose time course paralleled that of the increase in EPSP slope (figure 7.5.11). Owing to baseline changes in temperature occurring in both groups before the first infusion of ACPD, meaningful quantification of the data was impossible at this time point. However, temperature readings were fairly stable prior to the second ACPD infusion, allowing an estimate to be made of the expected rise in EPSP slope resulting from the observed increase in temperature.

The following analysis was carried out only on the 7 rats from each group that were passively warmed during the recording of evoked potentials. The increase in brain temperature, measured as the absolute change in mean value from the baseline recorded over the 10 min immediately prior to the second ACPD infusion, to the mean value over the 10 min following the end of infusion, did not differ between groups [PBS = 0.39 ± 0.09°C; MCPG = 0.34 ± 0.07°C; $F < 1$].

Figure 7.5.12 shows scatter plots relating EPSP slope to brain temperature, derived from the passive warming of rats at the end of each experiment. The slope of the function relating slope to temperature was fairly consistent across animals. Regression lines were calculated from each of the data sets shown in figure 7.5.12. The rise in EPSP slope expected following a temperature rise of 1 °C did not differ significantly between groups, indicating that EPSP slopes in both groups were equally sensitive to temperature changes [PBS = 19.75 ± 2.06 %; MCPG = 19.37 ± 2.29 %; $F < 1$]. It is worth noting that a previous study reported an EPSP slope increase of roughly 20 %, associated with a 1 °C increase in brain temperature (R. C. Rowan, unpublished observations). This is exactly the result that would be predicted based on the relationship between EPSP slope and brain temperature in the present study.

Based on the above data, the EPSP slope value expected to result from the observed temperature increase after the second ACPD infusion was calculated for each rat. No significant difference was found between the predicted rise in EPSP slope and the actual increase recorded (table 7.5.5). These

data are consistent with the possibility that the transient increases in EPSP slope after ACPD infusion are simply temperature artifacts. As expected, no significant overall effect of drug group (i.e. PBS vs. MCPG) was observed; no significant drug group x EPSP slope increase (i.e. observed or expected) was found (table 7.5.5).

Table 7.5.5 Increase in EPSP slope after the second ACPD infusion. Values expected on the basis of the temperature increase are compared with actual values recorded.

	Mean percentage increase in EPSP slope over the 10 min following the end of the 2nd ACPD infusion	
	Expected increase	**Observed increase**
PBS + 1 mM ACPD	7.60 ± 1.57	9.24 ± 1.76
200 mM MCPG + 1 mM ACPD	6.20 ± 1.33	6.06 ± 1.80

ANOVA result Overall difference between PBS and MCPG groups:	$F(1,12) = 1.38$; $p > 0.2$
Overall difference between observed and expected slope increases	$F < 1$
Drug group x slope increase (i.e. observed and expected) interaction	$F < 1$

A similar analysis was carried out on changes in population spike amplitude following the second infusion of ACPD. As before, the analysis was carried out only on the 7 rats from each group that were passively warmed during the recording of evoked potentials. Figure 7.5.13 shows scatter plots relating population spike amplitude changes to brain temperature, derived from the passive warming of rats at the end of each experiment. Regression lines were calculated from each of the data sets shown. The expected fall in population spike amplitude following a temperature rise of 1 °C did not differ significantly between groups [PBS = -0.97 ± 0.31 mV; MCPG = -0.59 ± 0.07mV; $F(1,12) = 1.38$; $p > 0.2$]. The fall in population spike amplitude expected to result from the observed temperature increase after the second ACPD infusion was calculated for each rat. However, population spike amplitudes in fact increased slightly after ACPD infusion. The difference between the expected fall in population spike amplitude and the observed rise was highly significant, indicating that the increase in population spike amplitude after ACPD infusion is not merely a

temperature artifact (table 7.5.6).

The overall difference between PBS and MCPG treated groups almost reached significance. However, no significant drug group by population spike change (i.e. observed or expected) was found. In fact, the observed population spike was approximately 0.7 mV larger than expected in both groups (table 7.5.6). The trend towards a group difference is merely an artifact of the slightly lower sensitivity of population spike amplitude to temperature changes in the 200 mM MCPG group (see above), coupled with the slightly lower temperature rise occurring in this group.

Table 7.5.6 Increase in population spike amplitude after the second ACPD infusion. Values expected on the basis of the temperature increase are compared with actual values recorded.

	Mean absolute change in population spike amplitude over the 10 min following the end of the 2nd ACPD infusion (mV)	
	Expected change	**Observed change**
PBS + 1 mM ACPD	-0.44 ± 0.17	0.22 ± 0.25
200 mM MCPG + 1 mM ACPD	-0.20 ± 0.04	0.51 ± 0.19

ANOVA result Overall difference between PBS and MCPG groups:	$F(1,12) = 4.59$; $0.1 > p > 0.05$
Overall difference between observed and expected population spike changes	$F(1,12) = 23.10$; $p < 0.001$
Drug group x population spike change (i.e. observed and expected) interaction	$F < 1$

7.5.4 Discussion

The finding that 10 µl 200 mM (*R,S*)-MCPG inhibits the rise in spontaneous activity induced by infusion of 1 mM and 4 mM (1*S*,3*R*)-ACPD confirms that the drug does indeed inhibit mGluRs whose activity increases spontaneous dentate noise. The blockade was equally effective at both 1 mM and 4 mM concentrations of ACPD. Note that the application of 4 mM (1*S*,3*R*)-ACPD / 200 mM (*R,S*)-MCPG reflects an agonist / antagonist ratio commonly used *in vitro* (e.g. 10 µM (1*S*,3*R*)-ACPD / 500 µM (*R,S*)-MCPG: Bashir et al., 1993). It therefore seems unlikely that the failure to block LTP in the two previous experiments was due to the failure of MCPG to inhibit mGluR activation.

Previous studies have demonstrated that mGluR activation can give rise to synchronized network oscillations within the hippocampus (see chapter 3.9.2). For instance, Whittington et al. (1995) found that application of (1*S*,3*R*)-ACPD elicited 40 Hz i.p.s.p. oscillations in area CA1. It was proposed that mGluR activation drives network oscillations of inhibitory interneurons, resulting in a 40 Hz rhythm that entrains the firing of pyramidal cells. Since the work presented in this chapter was carried out, Boddeke et al. (1997) have described a similar increase in CA1 spontaneous activity, with a dominant frequency of roughly 20 Hz. This rhythm was also found to be dependent on the activation of GABAergic interneurons. Although pharmacological characterization of the increase in spontaneous activity was not carried out in the present study, owing to the difficulties associated with such experiments *in vivo*, it is likely that the 20-50 Hz rhythm induced by ACPD in the dentate gyrus reflects a similar network oscillation to that described by Boddeke et al. (1997).

Although MCPG successfully antagonized the increase in spontaneous activity induced by ACPD, it unmasked a small dose-dependent depression (see figures 7.5.4B and 7.3.8B). This may reflect the action of ACPD at a subtype of mGluR not antagonized by MCPG. It is interesting that in cat visual cortex, ACPD either reduces or increases the level of spontaneous activity, depending on the cortical layer from which recordings are made (Reid and Daw, 1997). However, since the subtypes responsible for changes in spontaneous activity are unknown, and considering the controversy surrounding the actions of MCPG (see chapter 3.6.3 & 3.10), further speculation would be premature.

Infusion of ACPD caused only a transient increase in EPSP slope in the present study; lasting slow-onset potentiation of the kind described by Manahan-Vaughan and Reymann (1995a) was not observed. A slight rise in brain temperature was also associated with ACPD infusion (see chapter 3.11.2). Neither of these effects was blocked by the prior infusion of MCPG. In the 1 mM ACPD condition, at least, the increase in brain temperature was sufficient to account entirely for the observed slope increase. However, in contrast to the population spike decrease predicted by a temperature rise, ACPD infusion caused a clear increase in population spike after infusion of 4 mM ACPD (figure 7.5.2B). Although the rise in population spike was minimal after 1 mM ACPD infusion (figure 7.5.10B), spike amplitudes were still significantly larger than predicted by the temperature rise. Hence, whilst the increase in EPSP slope may be simply a temperature artifact, the rise in population spike cannot be accounted for in this way. This finding may be explained by an ACPD-induced decrease in the firing threshold of dentate granule cells, without an enhancement of fast synaptic transmission. Consistent with this possibility, it has been reported that application of ACPD can cause a depression of GABAergic transmission, resulting in an increase in CA1 pyramidal cell excitability. However, pharmacological isolation of AMPA receptor-mediated responses revealed that the fast EPSP was unaffected by ACPD application (Liu et al., 1993). It is possible that similar mechanisms underlie the transient increase in population spike in the present study.

The mGluR subtypes responsible for the various effects of ACPD are unknown, as is the subtype

responsible for the modulation of LTP. Hence, whilst these results demonstrate that MCPG blocks at least one subtype of (probably) hippocampal mGluR when infused intraventricularly, it is possible that actions on a different subtype explain the ability of MCPG to block LTP under some circumstances. This problem could potentially be solved by the use of subtype-specific antagonists. A further problem with the above assay is that MCPG exhibits greater potency in antagonizing ACPD-mediated responses, compared to those of glutamate (Littman and Robinson, 1994; Brabet et al., 1995; Huber et al., 1998). Again, this problem requires the use of novel antagonists that were not available when the present series of experiments was started.

Having established that MCPG does indeed antagonize mGluRs using the current experimental set-up (despite the qualifications raised above), the possibility that the mGluR activated molecular switch (see chapter 3.10.4) might provide an explanation for the failure of MCPG to block LTP was tested. This work is described in the following section.

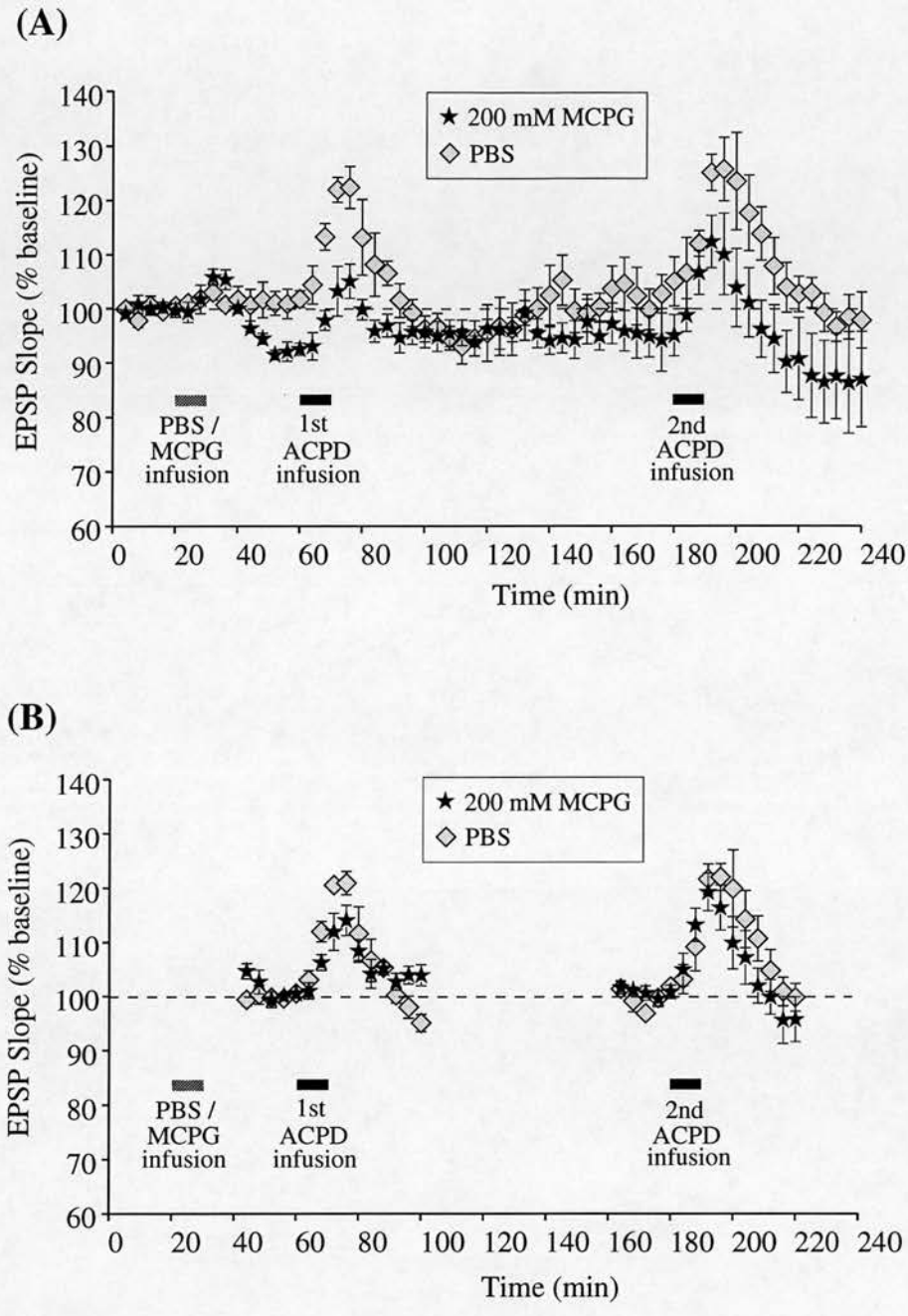


Fig. 7.5.1

Effect of 4 mM (1S,3R)-ACPD infusion on EPSP slope following the application of 200 mM (R,S)-MCPG or PBS. Data are normalized either to the 10 min prior to MCPG / PBS infusion (A), or to separate 10 min periods prior to each infusion of ACPD (B). Prior application of MCPG did not block the transient ACPD-induced rise in EPSP slope.

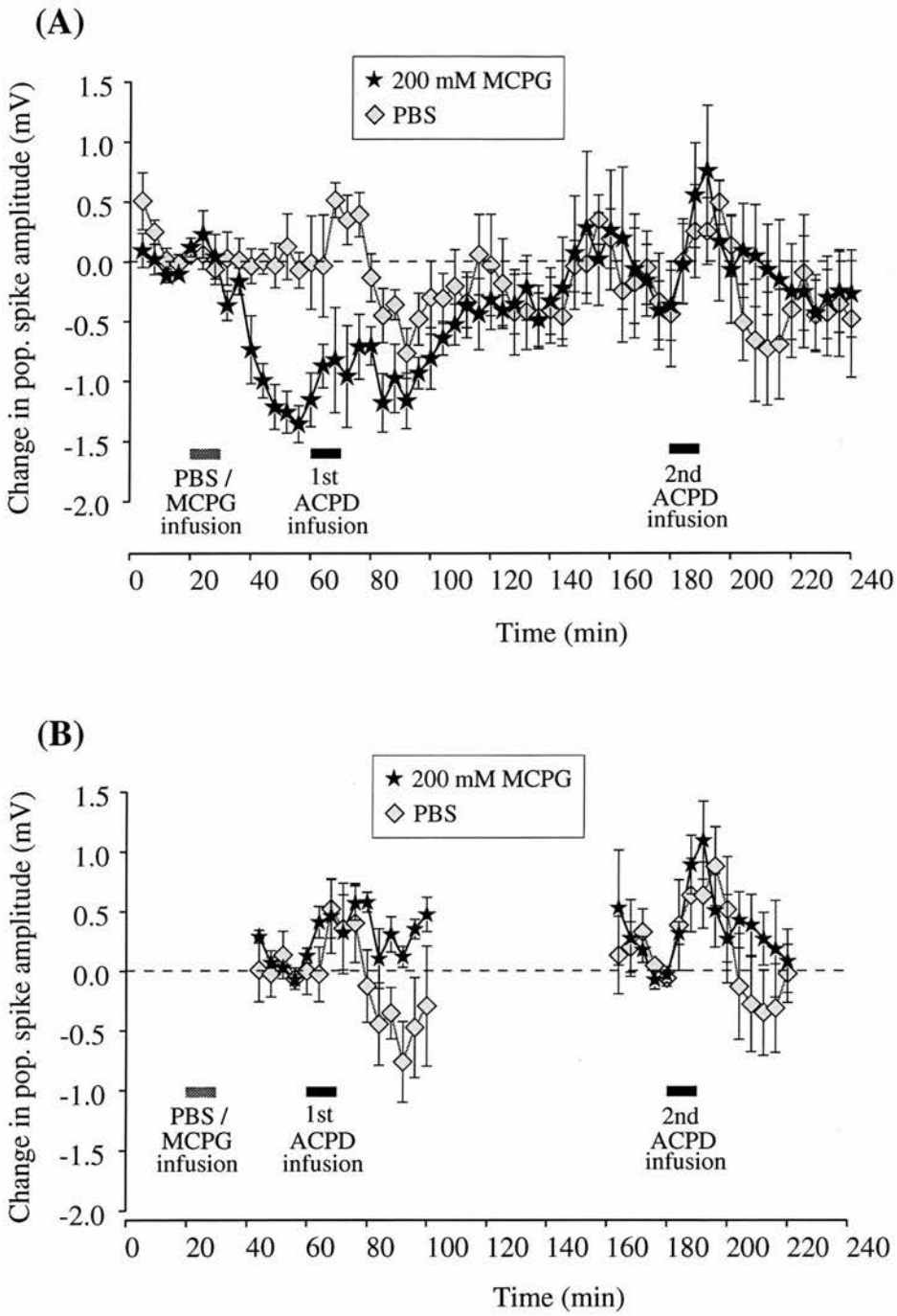


Fig. 7.5.2

Effect of 4 mM (1S,3R)-ACPD infusion on population spike amplitude after application of 200 mM (R,S)-MCPG or PBS. Data are normalized either to the 10 min prior to MCPG / PBS infusion (A), or to separate 10 min periods prior to each infusion of ACPD (B). Prior application of MCPG did not block the slight ACPD-induced rise in population spike amplitude.

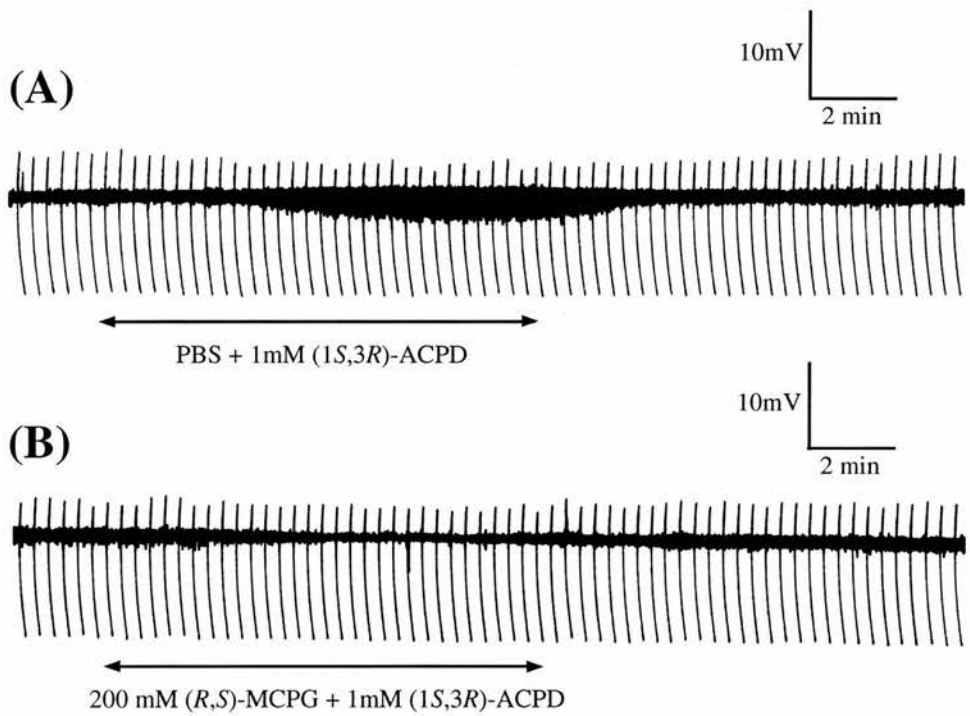


Fig. 7.3.3
Infusion of 1 mM (1*S*,3*R*)-ACPD induces a transient increase in spontaneous dentate activity when applied after buffered saline (A). This increase was completely blocked by prior application of 200 mM (*R*,*S*)-MCPG.

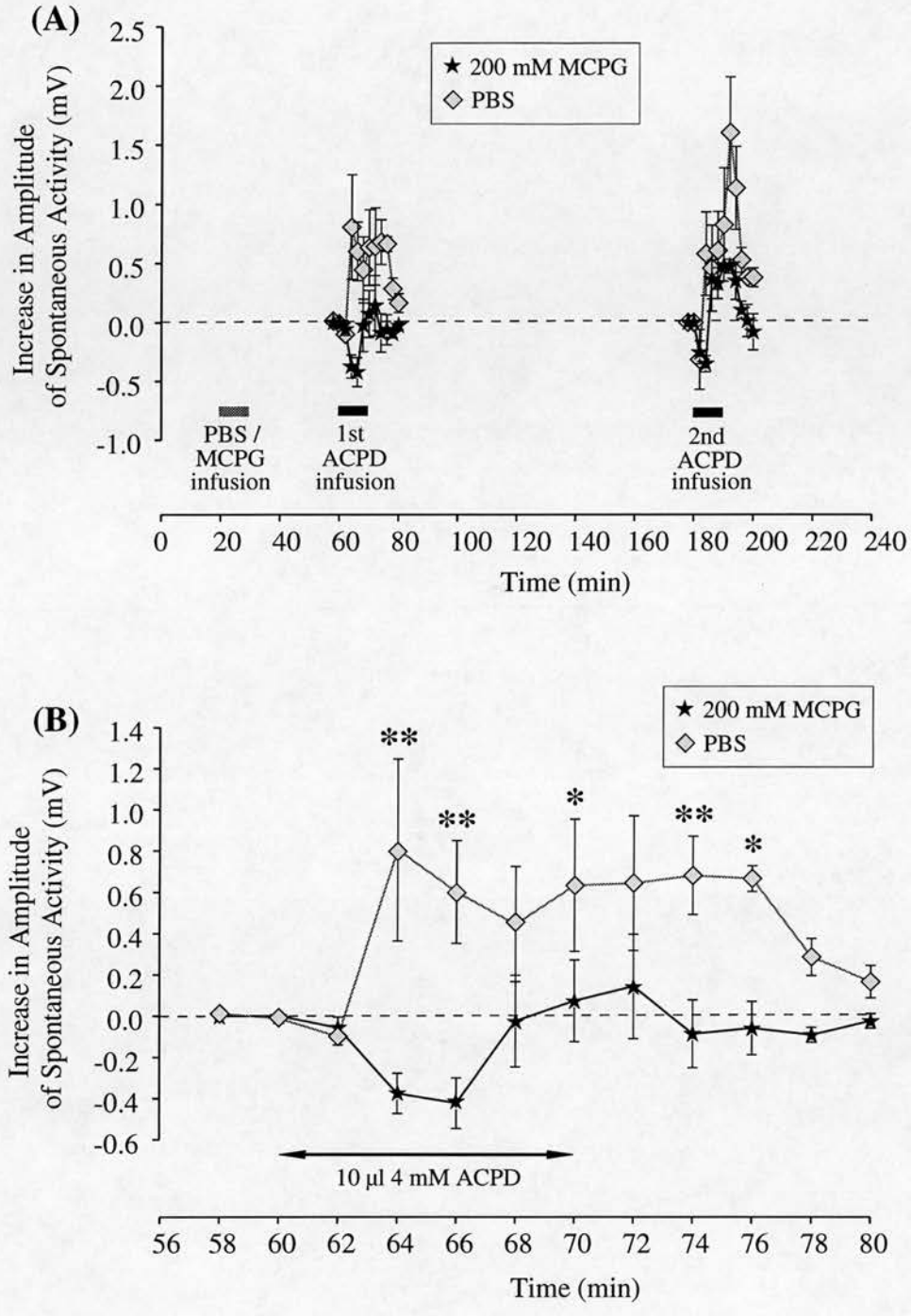


Fig. 7.5.4

(A) Mean increase in amplitude (peak to trough) of spontaneous activity resulting from infusion of 4 mM (1S,3R)-ACPD. Values were measured directly from polygraph chart records at 2 min intervals and normalized to the baseline level 0-2 min prior to the start of each individual infusion of ACPD. (B) Expanded display of the rise in spontaneous activity following the first infusion of ACPD. Prior application of 200 mM (R,S)-MCPG significantly reduced this effect (*$p < 0.05$; **$p < 0.01$).

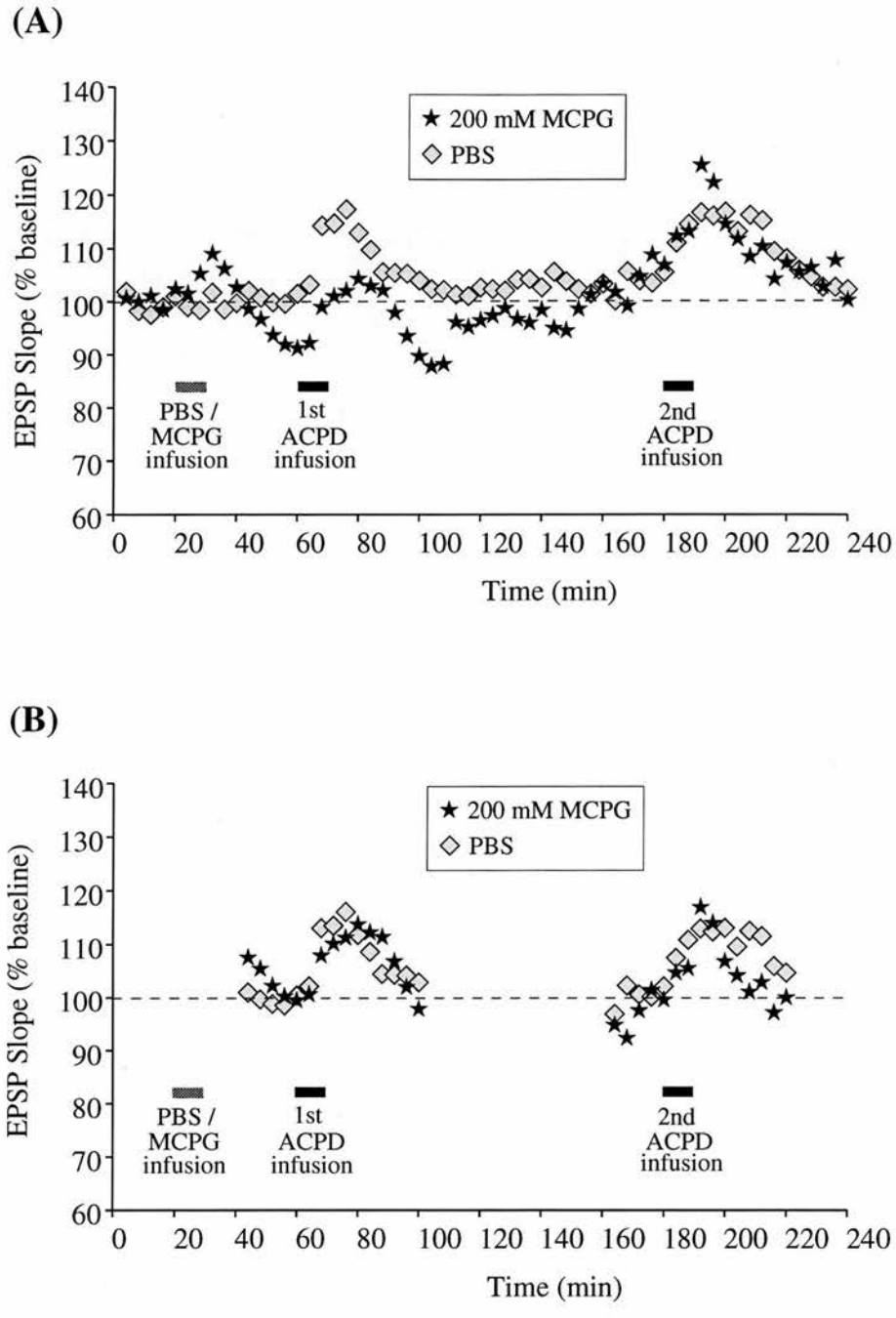


Fig. 7.5.5

Effect of 2 mM (1S,3R)-ACPD infusion on EPSP slope following the application of 200 mM (R,S)-MCPG (n = 1) or PBS (n = 1). Data are normalized either to the 10 min prior to MCPG / PBS infusion (A) or to separate 10 min periods prior to each infusion of ACPD (B). Application of MCPG did not block the transient ACPD-induced rise in EPSP slope.

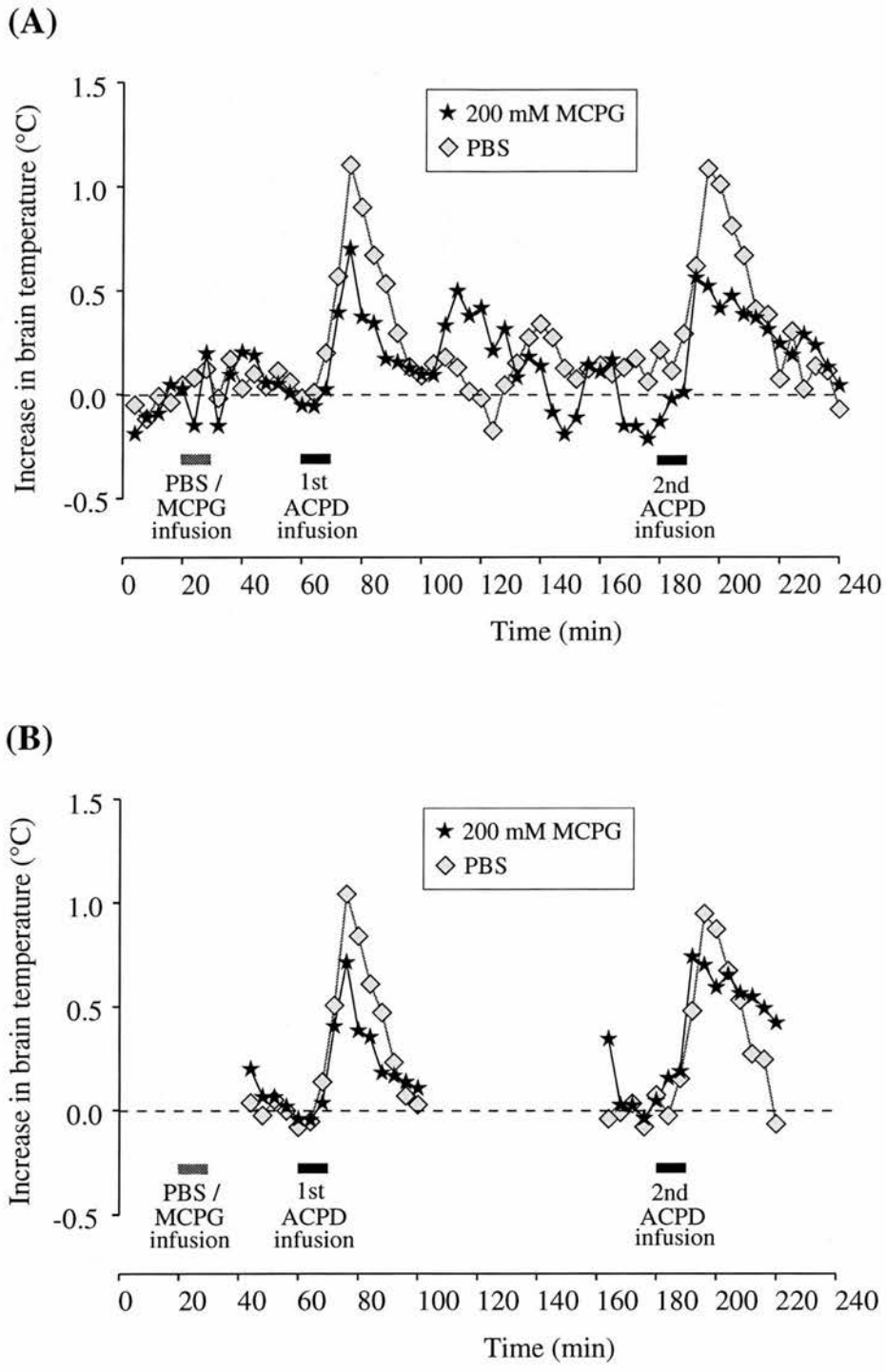


Fig. 7.5.6

Effect of 2 mM (1S,3R)-ACPD infusion on brain temperature. Data were normalized either to the 10 min prior to MCPG / PBS infusion (A), or to separate 10 min periods prior to each infusion of ACPD (B). Application of MCPG (n = 1) did not block the transient ACPD-induced temperature rise compared to the increase recorded after PBS infusion (n = 1).

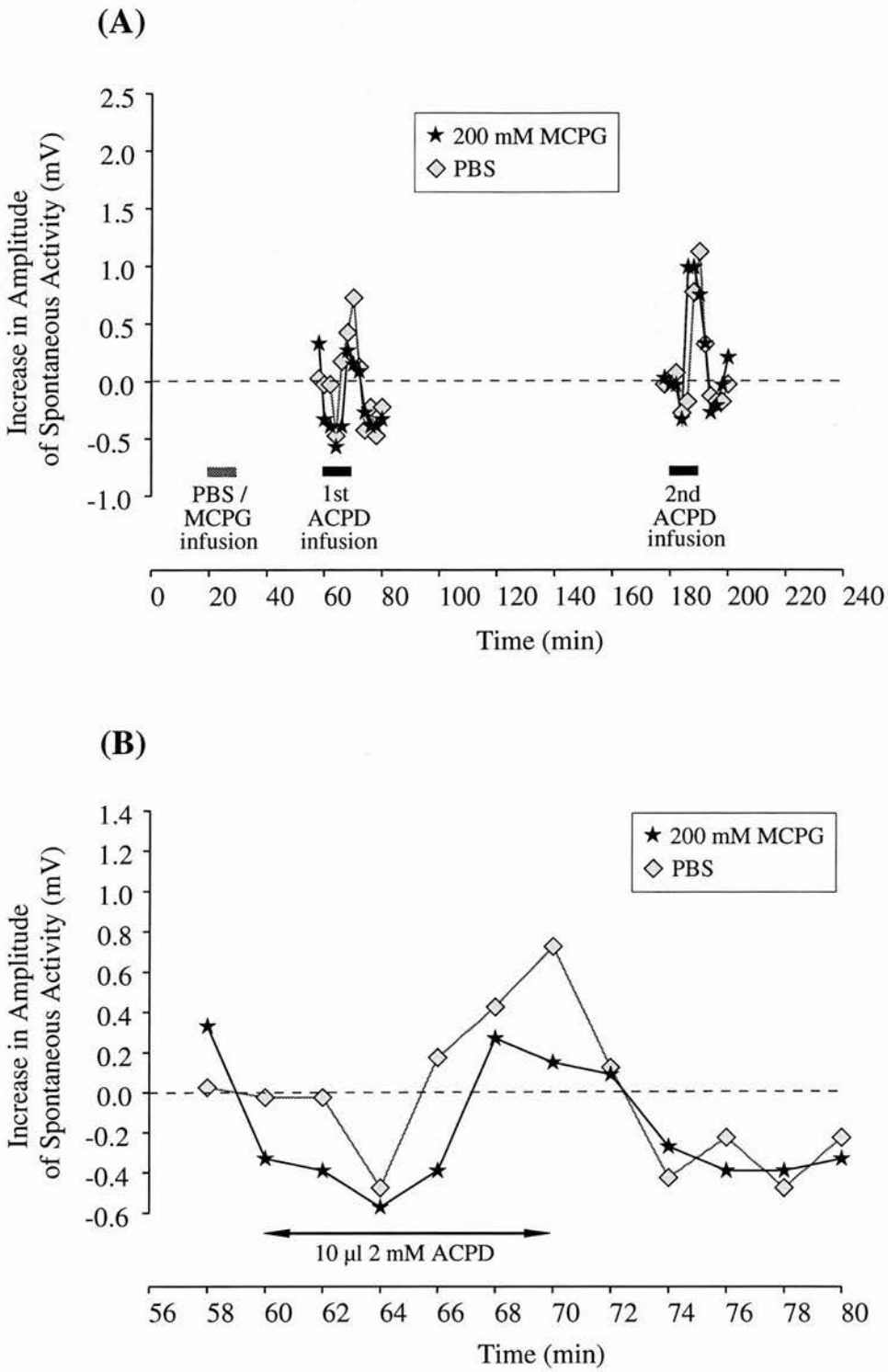


Fig. 7.5.7

(A) Mean increase in amplitude of spontaneous activity resulting from infusion of 2 mM (1S,3R)-ACPD. (B) Expanded display of the rise in spontaneous activity following the first infusion of ACPD.

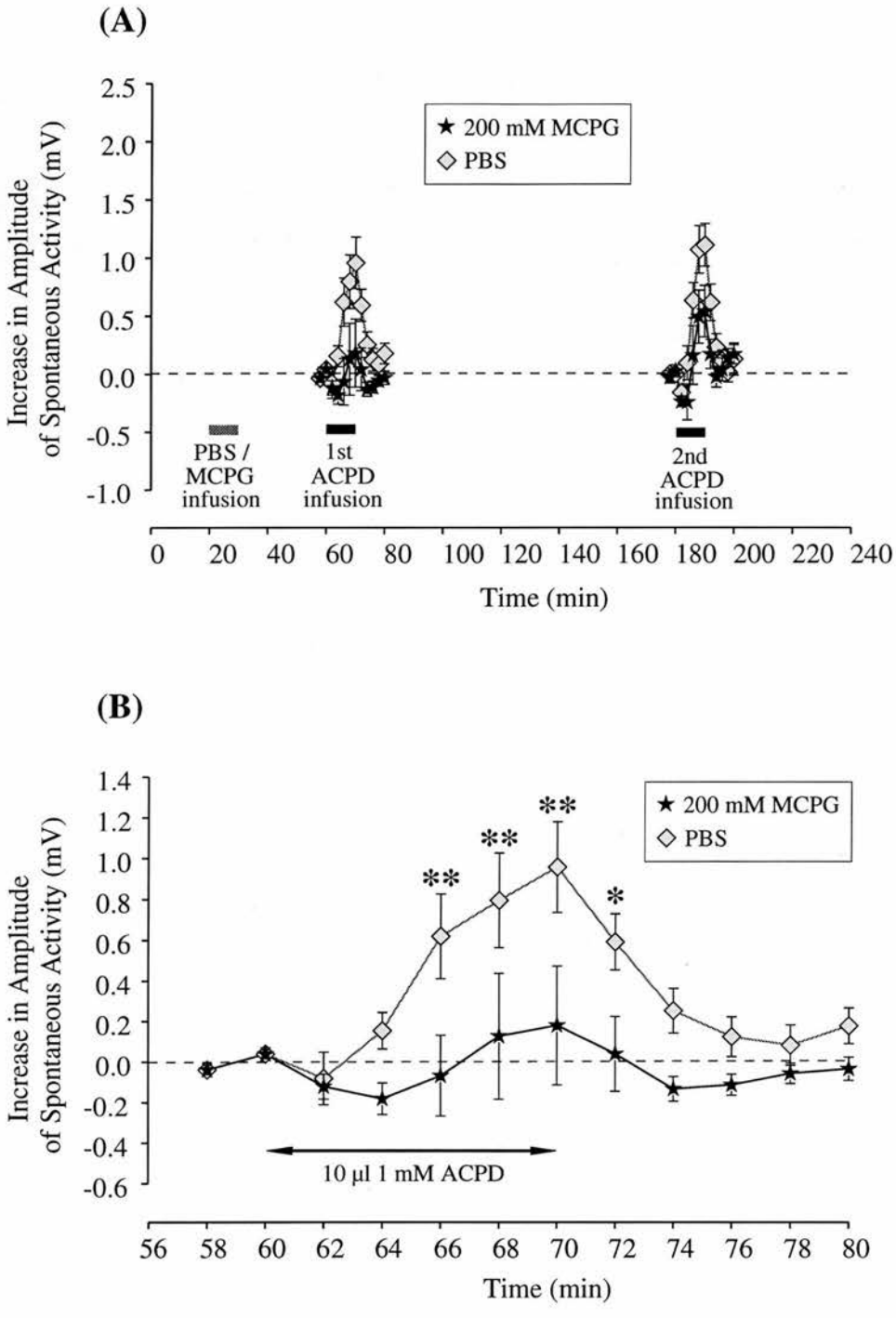


Fig. 7.5.8

(A) Mean increase in amplitude (peak to trough) of spontaneous activity resulting from infusion of 1 mM (1S,3R)-ACPD. (B) Expanded display of the rise in spontaneous activity following the first infusion of ACPD. Prior application of 200 mM (R,S)-MCPG significantly reduced this effect (*$p < 0.05$; **$p < 0.01$).

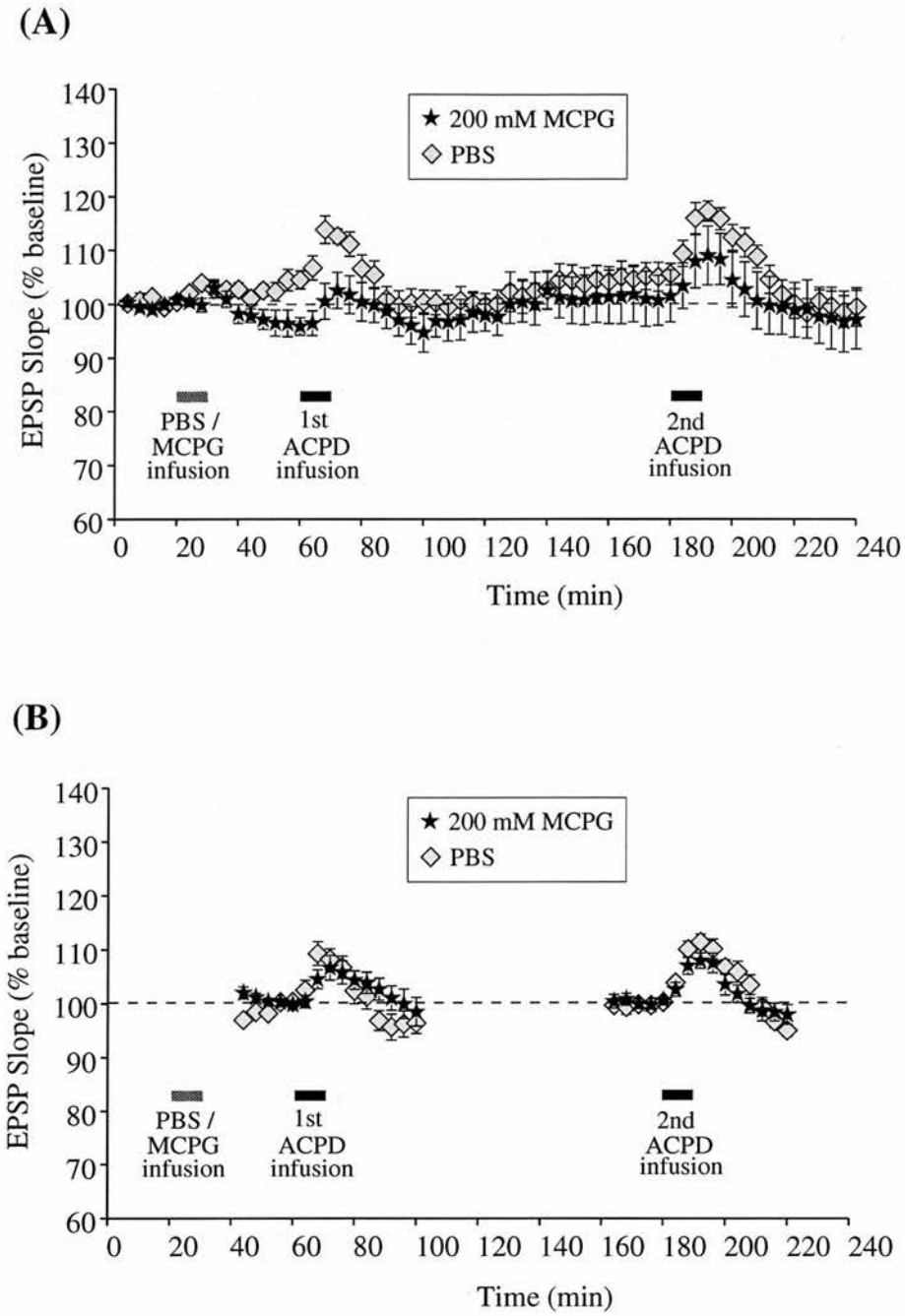


Fig. 7.5.9

Effect of 1 mM (1S,3R)-ACPD infusion on EPSP slope following the application of 200 mM (R,S)-MCPG or PBS. Data are normalized either to the 10 min prior to MCPG / PBS infusion (A), or to separate 10 min periods prior to each infusion of ACPD (B). Application of MCPG did not block the transient ACPD-induced rise in EPSP slope.

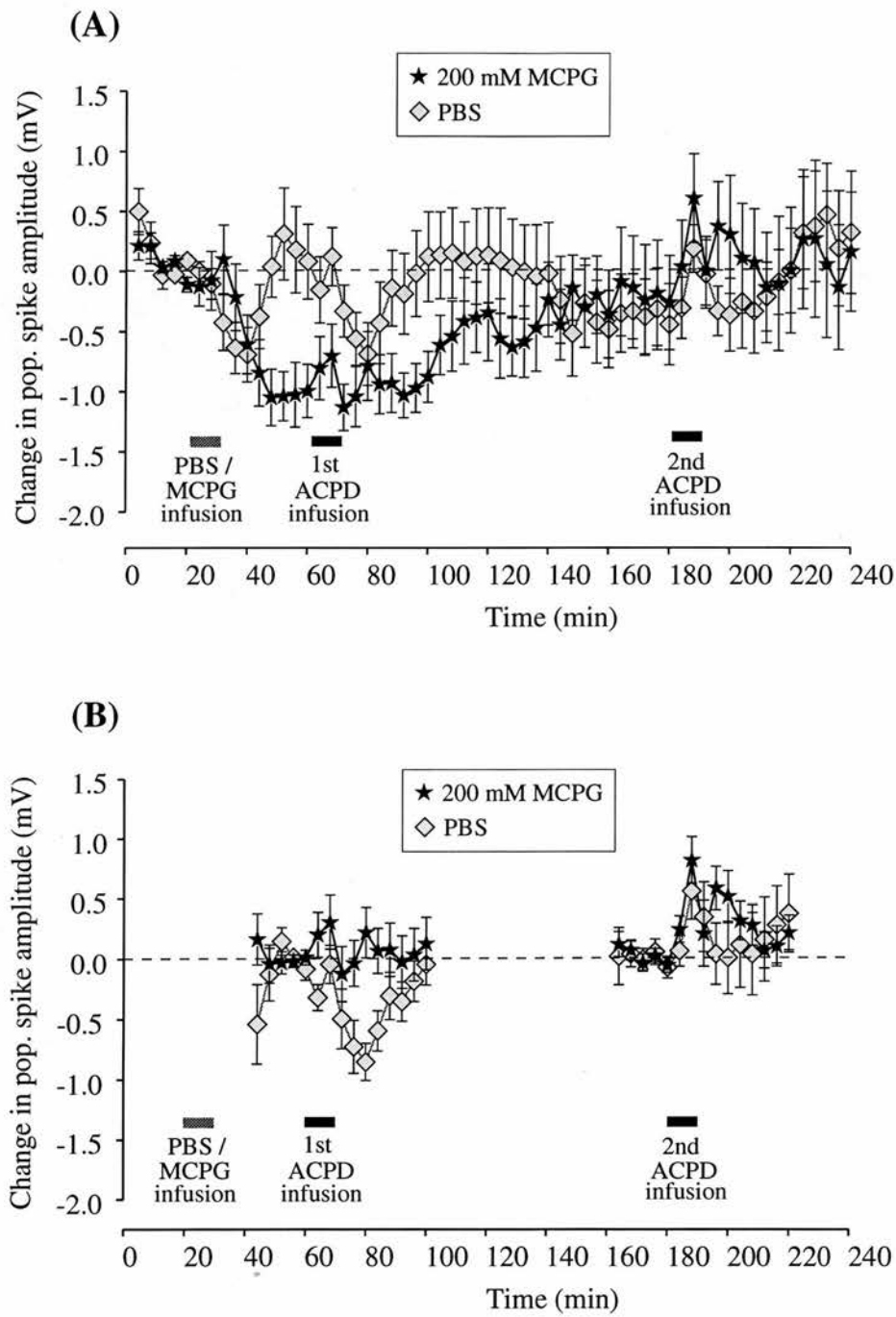


Fig. 7.5.10

Effect of 1 mM (1S,3R)-ACPD infusion on population spike amplitude after application of 200 mM (R,S)-MCPG or PBS. Data are normalized either to the 10 min prior to MCPG / PBS infusion (A), or to separate 10 min periods prior to each infusion of ACPD (B). Application of MCPG did not block the slight ACPD-induced rise in population spike amplitude.

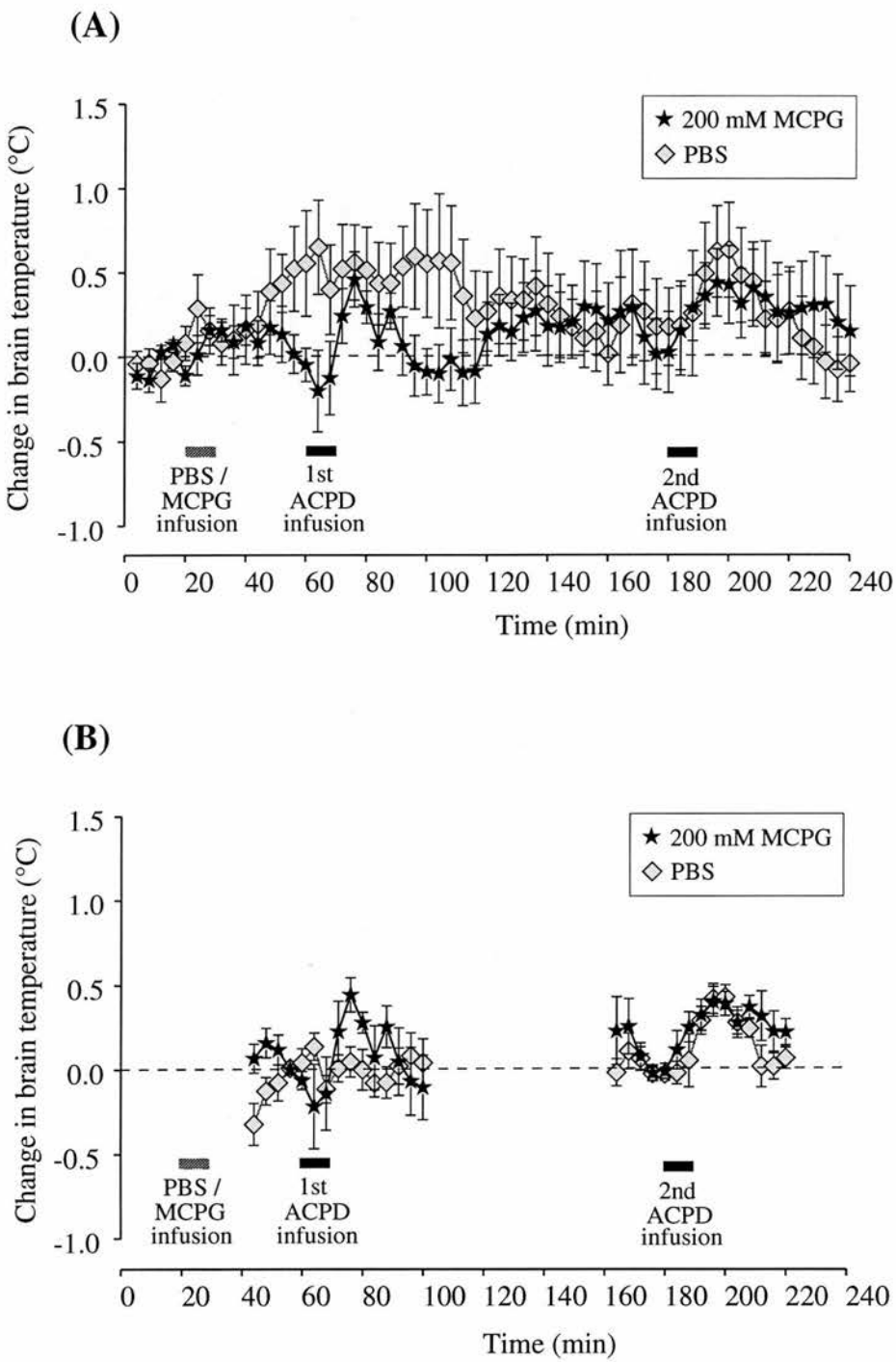


Fig. 7.5.11

Effect of 1 mM (1S,3R)-ACPD infusion on brain temperature. Data were normalized either to the 10 min prior to MCPG / PBS infusion (A), or to separate 10 min periods prior to each infusion of ACPD (B). Application of MCPG did not block the transient ACPD-induced temperature rise.

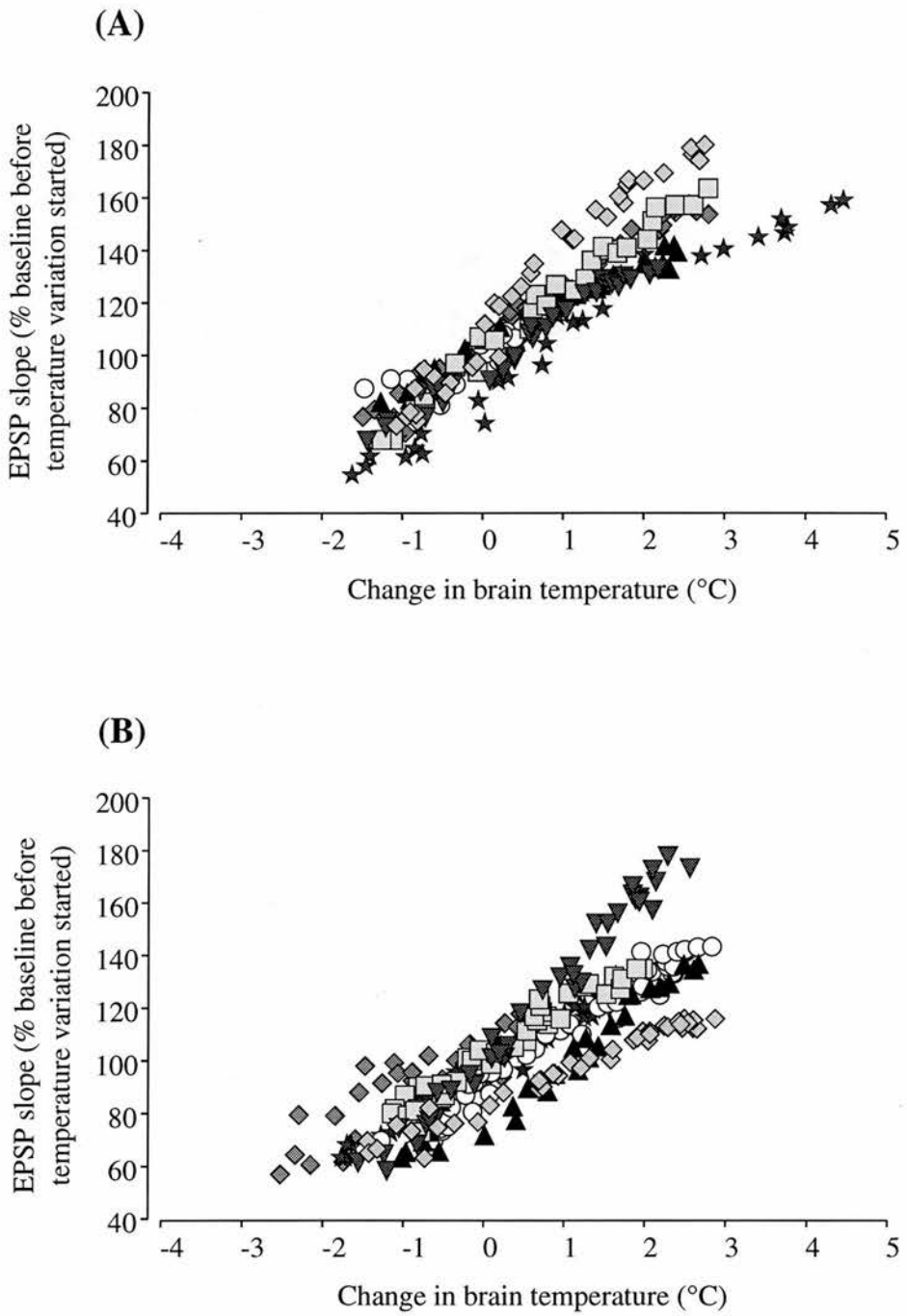


Fig. 7.5.12

Relationship between EPSP slope and brain temperature during passive warming of animals (1 mM (1S,3R)-ACPD study). Data from the PBS-treated group are plotted in part (A). Data from the 200 mM (R,S)-MCPG-treated group are plotted in part (B). The different symbols correspond to data obtained from individual rats.

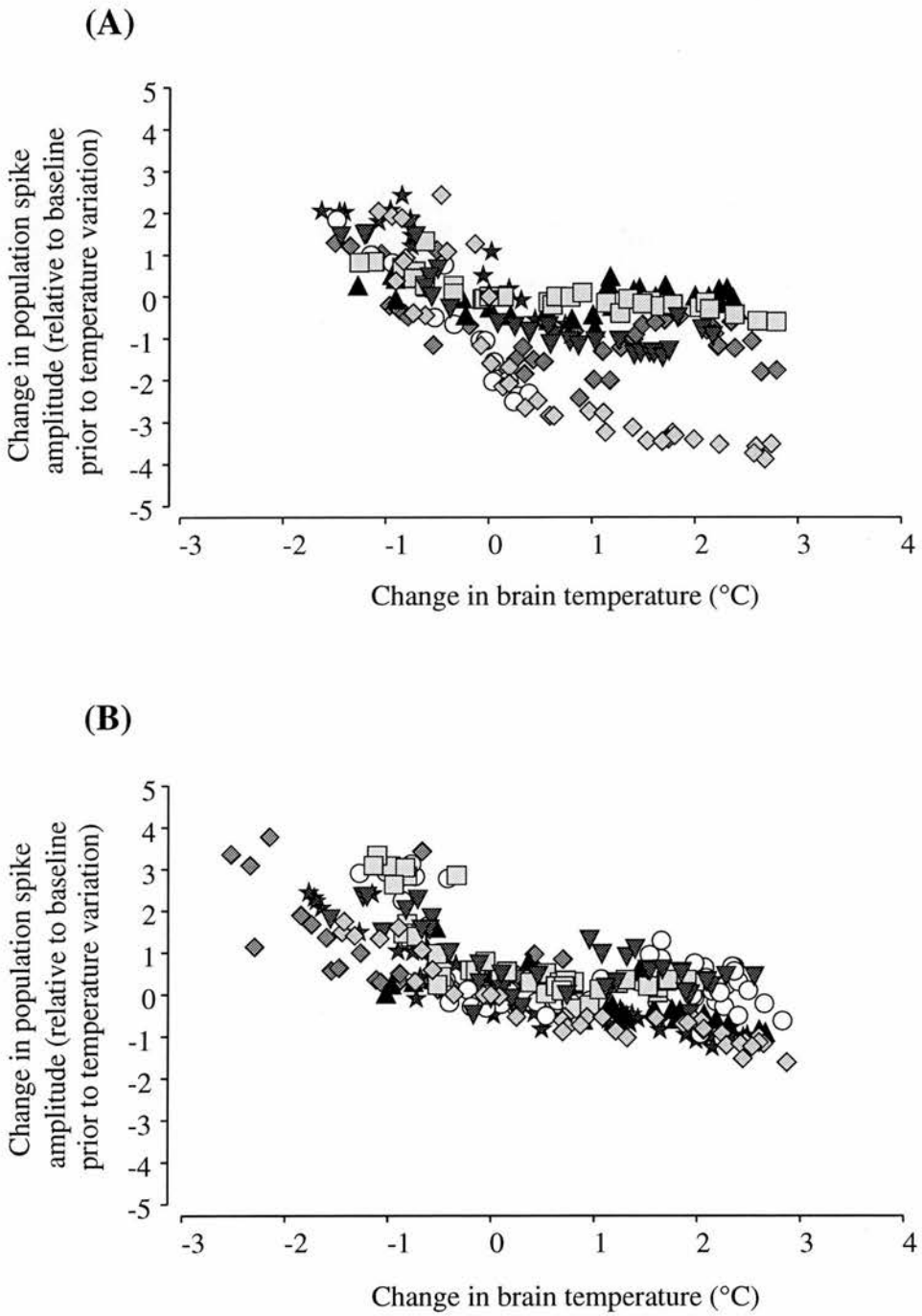


Fig. 7.5.13

Relationship between population spike amplitude and brain temperature during passive warming of animals (1 mM (1S,3R)-ACPD study). Data from the PBS-treated group are plotted in part (A). Data from the 200 mM (R,S)-MCPG-treated group are plotted in part (B). Symbols correspond to data obtained from individual rats.

7.6 Does prior depotentiation reveal an MCPG-sensitive component of LTP in vivo?

7.6.1 Introduction

One potential explanation for the mixed results obtained with MCPG is provided by the results of Bortolotto et al. (1994), who postulated that mGluR activation turns on a molecular switch that eliminates the requirement for further mGluR activation in the induction of LTP. This switch was found to be turned off by the delivery of low frequency stimulation. Wang et al. (1995) subsequently reported that even without deliberate activation of mGluRs (e.g. by tetanic stimulation or agonist application), a block of LTP with MCPG was only obtained after low frequency stimulation of afferents. It is possible that under some circumstances, the mGluR-activated molecular switch may be turned on prior to the start of an experiment, thus abolishing the ability of MCPG to block LTP, a possibility that has not previously been investigated *in vivo*.

The following experiments were conducted in an attempt to address this issue. However, rather than delivering low frequency stimulation under baseline conditions, it was decided to follow the Bortolotto et al. (1994) protocol in which LTP is first induced, then depotentiated using low frequency stimulation prior to drug infusion and further tetanization. This allows the efficacy of low frequency stimulation in reversing LTP to be judged, in addition to its actions on the putative molecular switch.

It has been reported that the percentage reversal of LTP in CA1 slices declines steeply as the interval between tetanus and low-frequency stimulation is increased (Stäubli and Chun, 1996). Owing to this finding, and since low-frequency stimulation does not readily reverse stable LTP in the dentate gyrus, it was decided that the depotentiating train should be delivered very soon after tetanization. Hence, low frequency stimulation was started 2 min after the final tetanus train. A range of different frequencies have been reported to induce long-term depression or reverse LTP, but 5 Hz stimulation (the frequency chosen in the present study) has generally been found to be optimal for inducing depotentiation *in vivo* (see chapter 8).

7.6.2 Methods

The recording set-up was identical to that used in experiment 7.3. A 20 min baseline period was recorded, followed by a high frequency tetanus. However, 2 min after tetanization, a 1 min period of 5 Hz stimulation was applied to most rats. One hour after the first tetanus, these animals were infused with either 200 mM (*R,S*)-MCPG (n = 7) or vehicle (n = 8). Half an hour after the end of drug infusion, a second tetanus was given, and LTP was followed for 1 hr. A control group (n = 8) also

received the first tetanus, but no depotentiating stimulation. LTP was followed for one hour in these animals, and their experiment ended at the point when drug infusion would have begun for the other groups.

In a follow-up study, the period of 5 Hz stimulation was increased to 10 min. All groups consisted of 6 animals in this experiment.

7.6.3 Results

7.6.3.1 1 min 5 Hz

Tetanus 1 and depotentiation

Figure 7.6.1A shows EPSP slope data recorded throughout the experiment, normalized to the mean value over the 10 min prior to the first tetanus. The change in population spike amplitude is shown in figure 7.6.1B. Significant depotentiation was induced by 5 Hz stimulation given 2 min after tetanization. An ANOVA of the amount of EPSP slope potentiation 50-60 min after the first tetanus, in which all depotentiated animals were compared with controls, revealed a significant reduction in LTP. A non-significant trend towards a reduction in population spike amplitude was also observed (table 7.6.1). However, the depotentiation was incomplete: only 2 of the 15 rats receiving 5 Hz stimulation showed less than 5 % EPSP slope LTP after 1 hr, in contrast to control rats, all of which showed more than 5 % LTP.

Owing to the 2 min interval between tetanus and 5 Hz stimulation, PTP was measured over the period between 0-2 min after tetanization (rather than 0-4 min as in 7.3 & 7.4). No significant group differences were found in the amount of EPSP or population spike PTP 0-2 min post-tetanus (table 7.6.1).

Table 7.6.1 Percentage potentiation induced by a high frequency tetanus followed by a 1 min period of 5 Hz stimulation.

	EPSP slope (% baseline)		**Increase in pop. spike amplitude (mV)**	
	0-2 min post-tetanus (PTP)	**50-60 min post tetanus (LTP)**	**0-2 min post-tetanus (PTP)**	**50-60 min post tetanus (LTP)**
Tetanus only	151.2 ± 2.4	120.2 ± 3.4	4.71 ± 0.62	2.78 ± 0.35
Tetanus + 5 Hz	150.4 ± 2.4	111.5 ± 1.9	5.21 ± 0.41	2.02 ± 0.21
ANOVA results	$F < 1$	$F(1,21) = 5.87$; $p < 0.05$	$F < 1$	$F(1,21) = 3.84$; $0.1 > p > 0.05$

No significant group differences were found in baseline stimulation intensity, initial spike amplitude over the 10 min prior to tetanus 1, and absolute slope magnitude prior to tetanus 1 (table 7.6.2).

Table 7.6.2 Baseline parameters.

	Stimulation Intensity (μA)	**Initial EPSP slope (mV/ms)**	**Initial pop. spike amplitude (mV)**
Tetanus only	288.8 ± 34.5	3.18 ± 0.12	2.86 ± 0.21
Vehicle	300.0 ± 26.6	3.40 ± 0.23	2.79 ± 0.13
200 mM (*R,S*)-MCPG	255.7 ± 11.7	3.77 ± 0.27	3.18 ± 0.23
ANOVA results	$F < 1$	$F(2,20) = 2.02$; $p > 0.1$	$F(2,20) = 1.19$: $p > 0.3$

Drug infusion and tetanus 2

Infusion of MCPG caused a marked fall in baseline slope values (see section 7.6.4), but failed to block LTP after the second tetanus (see figure 7.6.1A). Similarly, MCPG infusion did not affect population spike LTP following the second tetanus.

However, since depotentiation was not completely effective, it was considered more appropriate to re-normalize the data to the elevated baseline after the first tetanus. The baseline fall induced by MCPG infusion introduces a further complication: should data be normalized to the baseline immediately prior to the second tetanus, or to the baseline prior to drug infusion? Since the MCPG induced baseline fall appears to be transient (see experiment 7.4), the pre-infusion baseline would seem to be most appropriate. Table 7.6.3 shows the amount the amount of EPSP slope and population spike LTP 50-60 min after the second tetanus, relative to both pre-infusion and pre-tetanus baselines. When normalized to the pre-infusion baseline, no group differences in EPSP slope or population spike LTP were found.

However, when normalized to baseline immediately prior to the second tetanus, MCPG-treated animals displayed significantly more LTP than vehicle-infused controls. This result is an artifact of falling pre-tetanus baselines in the former group, leading to an artificially elevated measure of LTP. It is curious that the baseline fall in EPSP slope appears more pronounced when MCPG is infused during the recording of a potentiated baseline (figure 7.6.1A) compared to that obtained in a non-potentiated baseline (figures 7.3.1A and 7.4.1A). This phenomenon is discussed in section 7.7.

Table 7.6.3 Potentiation following a second tetanus delivered after infusion of 200 mM (*R,S*)-MCPG or PBS.

	EPSP slope (% baseline)		**Increase in pop. spike amplitude (mV)**	
	relative to pre-*tetanus* baseline	**relative to pre-*infusion* baseline**	**relative to pre-*tetanus* baseline**	**relative to pre-*infusion* baseline**
PBS	114.1 ± 4.2	114.2 ± 2.6	2.17 ± 0.40	1.89 ± 0.46
200 mM (*R,S*)-MCPG	125.0 ± 5.6	114.5 ± 6.2	3.61 ± 0.25	2.77 ± 0.24

ANOVA results	$F(1,13) = 3.21$; $p < 0.05$	$F < 1$	$F(1,13) = 8.57$; $p < 0.05$	$F(1,13) = 2.59$; $p > 0.1$

The above results demonstrate that MCPG does not block LTP after significant depotentiation has previously been induced. However, depotentiation was only partial, suggesting that deconditioning of the molecular switch, if induced, may also have been incomplete (see section 7.6.4). It remains possible that a more effective depotentiation protocol might produce a different result. Hence, the period of 5 Hz stimulation was increased from 1 min to 10 min.

7.6.3.2 10 min 5 Hz

Tetanus 1 and depotentiation

Figure 7.6.2A shows EPSP slope data recorded throughout the experiment normalized to the mean value over the 10 min prior to the first tetanus. The change in population spike amplitude is shown in figure 7.6.1B. An ANOVA of the amount of EPSP slope LTP 50-60 minutes after tetanus 1, in which all depotentiated animals (n = 12) were compared with controls (n =6), revealed a highly significant reduction in the depotentiated group (table 7.6.4). No significant differences in the amount of PTP 0-2 min post-tetanus were found. An analysis of population spike depotentiation gave similar results.

Table 7.6.4 Percentage potentiation induced by a high frequency tetanus followed by a 10 min period of 5 Hz stimulation.

	EPSP slope (% baseline)		Increase in pop. spike amplitude (mV)	
	0-2 min post-tetanus (PTP)	**50-60 min post tetanus (LTP)**	**0-2 min post-tetanus (PTP)**	**50-60 min post tetanus (LTP)**
Tetanus only	150.5 ± 4.6	119.3 ± 1.4	4.86 ± 0.67	2.84 ± 0.19
Tetanus + 5 Hz	150.6 ± 2.4	105.6 ± 2.5	5.39 ± 0.56	0.87 ± 0.20
ANOVA results	$F < 1$	$F(1,16) = 13.3$; $p < 0.01$	$F < 1$	$F(1,16) = 38.6$; $p < 0.0001$

No group differences were found in baseline stimulation intensity, initial spike amplitude over the 10 min before tetanus 1, and absolute slope magnitude over the 10 min before tetanus 1 (table 7.6.5).

Table 7.6.5 Baseline parameters.

	Stimulation Intensity (μA)	**Initial EPSP slope (mV/ms)**	**Initial pop. spike amplitude (mV)**
Tetanus only	283.3 ± 45.9	3.83 ± 0.27	3.15 ± 0.40
Vehicle	313.3 ± 38.8	4.08 ± 0.25	2.85 ± 0.20
200 mM MCPG	241.7 ± 30.0	3.66 ± 0.24	3.25 ± 0.24
ANOVA results	$F < 1$	$F < 1$	$F < 1$

This protocol was slightly more effective than that used previously. A comparison of the amount of EPSP slope LTP following 5 Hz stimulation for 1 min and the amount following 10 min revealed a trend towards greater EPSP slope depotentiation in the latter (table 7.6.6). Furthermore, population spike depotentiation was considerably more effective after 10 min of 5 Hz, compared to 1 min of 5 Hz (table 7.6.6). EPSP slope values returned to within 5 % of baseline or less in 7 of the 12 rats given 10 min of 5 Hz, compared to only 2 out of 15 given 1 minute of low frequency stimulation. No significant differences in non-depotentiated control LTP were seen between the 1 min of 5 Hz and 10 min of 5 Hz studies (table 7.6.6); all controls showed more than 5 % LTP.

Table 7.6.6 Residual LTP after 1 min of 5 Hz compared to that remaining after 10 min of 5 Hz.

	EPSP slope LTP 50-60 min after first tetanus (% baseline)	**Increase in population spike amplitude 50-60 min after first tetanus (mV)**
1 min 5 Hz	111.5 ± 1.9	2.02 ± 0.21
10 min 5 Hz	105.6 ± 2.5	0.87 ± 0.20
ANOVA results	$F(1,25) = 3.59$; $0.1 > p > 0.05$	$F(1,25) = 14.6$; $p < 0.001$

Drug infusion and tetanus 2

MCPG did not block LTP of either the EPSP slope (figure 7.6.2A) or population spike (figure 7.6.2B) following the second tetanus, despite substantial depotentiation of previously induced LTP. This was true even in individual cases where 5 Hz stimulation returned EPSP slope levels to the original baseline, prior to drug infusion and further tetanization. An example of such a recording is shown in figure 7.6.3.

MCPG still did not block LTP as measured relative to the 10 min prior to drug infusion, or the 10 min prior to the second tetanus (table 7.6.7). In fact, whereas EPSP slope LTP did not differ between MCPG and PBS groups when normalized to the pre-infusion baseline, the population spike increase was significantly higher in the MCPG group. The reason for this finding is unknown, although the small group sizes may provide an explanation. As in the 1 min of 5 Hz study, both EPSP slope and population spike LTP were significantly higher in the MCPG group when normalized to the baseline immediately before the second tetanus. This result is explained by falling baselines induced by MCPG infusion.

These above results confirm the original finding that the inability of MCPG to block LTP is unaffected by prior depotentiation.

Table 7.6.7 Potentiation following a second tetanus delivered after infusion of 200 mM (*R*,*S*)-MCPG or PBS.

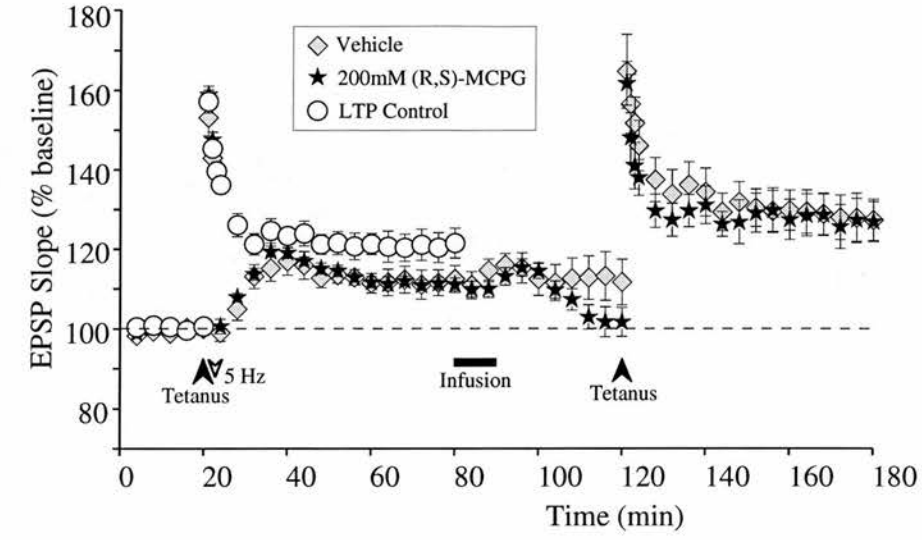
	EPSP slope (% baseline)		**Increase in pop. spike amplitude (mV)**	
	relative to pre-*tetanus* baseline	**relative to pre-*infusion* baseline**	**relative to pre-*tetanus* baseline**	**relative to pre-*infusion* baseline**
PBS	110.0 ± 3.9	108.3 ± 5.5	2.44 ± 0.33	2.03 ± 0.22
(*R*,*S*)-MCPG	121.2 ± 2.9	114.8 ± 1.44	4.59 ± 0.87	3.44 ± 0.55
ANOVA results	$F(1,10) = 5.27$; $p < 0.05$	$F(1,10) = 1.31$; $p > 0.2$	$F(1,10) = 5.36$; $p < 0.05$	$F(1,10) = 5.56$; $p < 0.05$

7.6.4 Discussion

Depotentiating stimulation was successful in reversing LTP, but did not re-establish an ability of MCPG to block LTP following a second tetanus. It is conceivable that LTP reaches a ceiling after the second tetanus thus masking differences in potentiation between the groups. However, this explanation is unlikely. The EPSP slope 1 hr after tetanus 2 in figure 7.6.2A was a mere 20 % above the initial baseline level recorded prior to tetanus 1, an implausibly low level for an LTP "ceiling".

The failure of prior depotentiation to restore the ability of MCPG to block LTP is consistent with the findings of Selig et al. (1995) and Thomas and O'Dell (1995) who failed to replicate the molecular switch findings of Bortolotto and Collingridge (1994) in area CA1 *in vitro*. However, there may be important differences between the reversal of established LTP *in vivo* with 1 Hz stimulation, and the "destabilization" of potentiation within minutes of its induction *in vivo*, using 5 Hz stimulation (see chapter 8.5.3). For instance, although not routinely recorded, there was evidence of epileptiform activity during 5 Hz stimulation in the present study, a finding not associated with stimulation at 1 Hz (see experiment 8.4). However, the mechanistic relationship between depotentiation and "deconditioning" of the mGluR activated molecular switch is unclear, although both are induced by low-frequency stimulation (Bortolotto and Collingridge, 1994). Under these circumstances, the importance of possible differences between the form of depotentiation described here and that typically induced in slice preparations is hard to assess. Furthermore, differences between the CA1 *in vitro* and dentate gyrus *in vivo* preparations may contribute to the difference in results obtained. Hence, although providing no support for the hypothesis, the results of the present study do not rule out the possibility that the sensitivity of LTP to MCPG may, under certain circumstances, be governed by the position of a molecular switch.

(A)



(B)

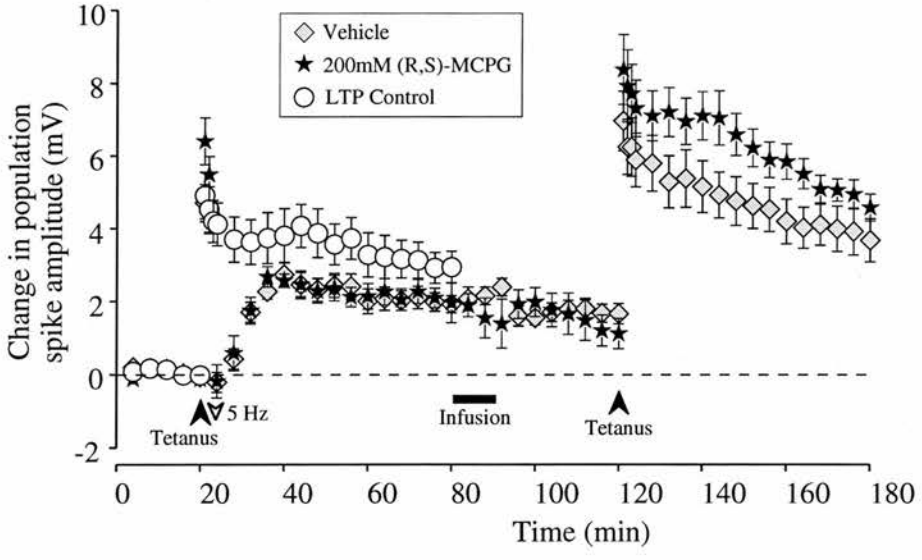


Fig. 7.6.1

Low frequency stimulation (5 Hz for 1 min) resulted in a significant reduction in both EPSP slope LTP (A), and population spike LTP (B), relative to controls receiving only a high frequency tetanus. Infusion of MCPG did not prevent the LTP of either measure following a second tetanus.

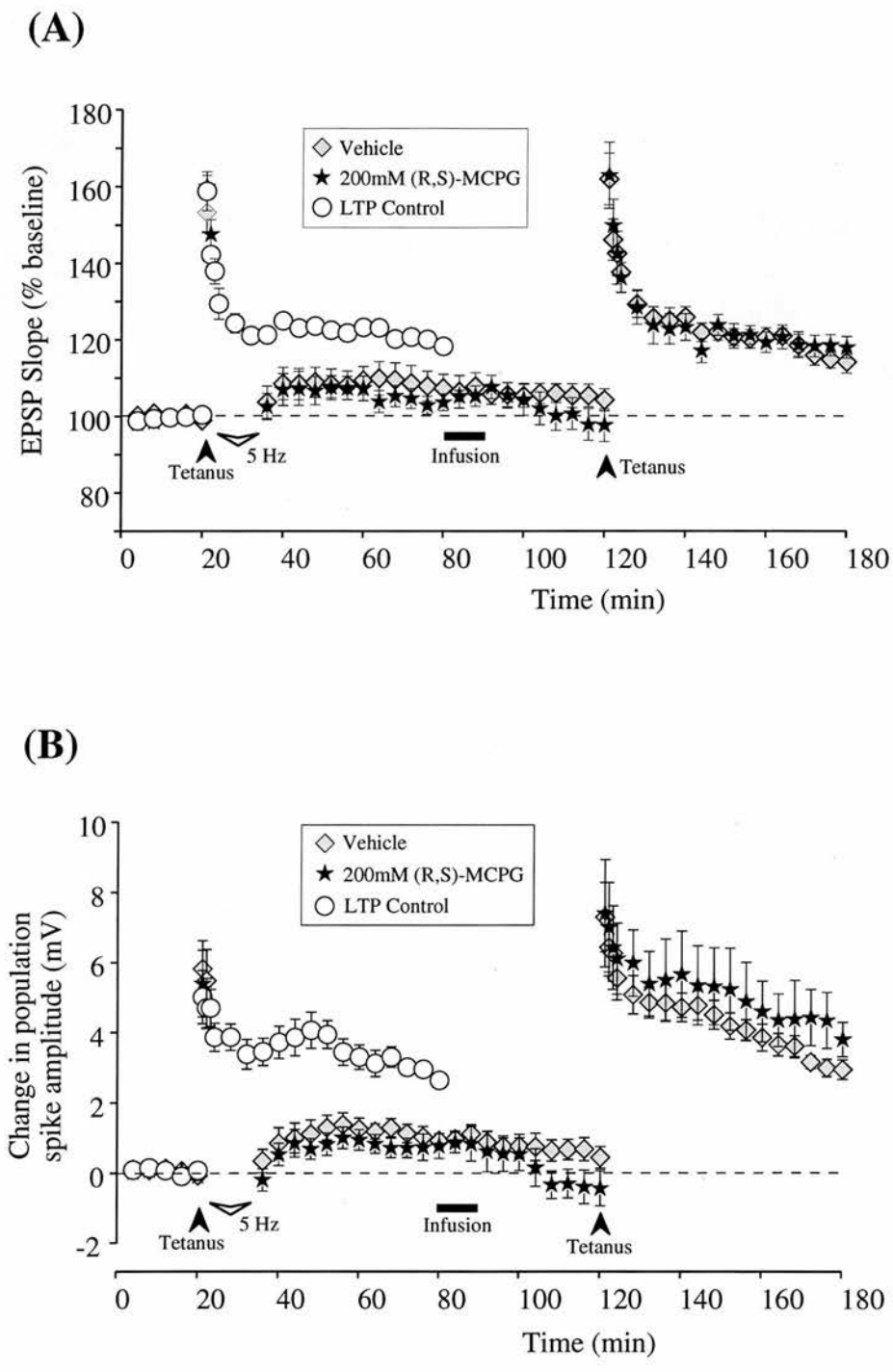


Fig. 7.6.2

10 min of 5 Hz stimulation resulted in a slightly more effective reversal of LTP than 1 min of 5 Hz. However, MCPG still did not block LTP of the EPSP slope (A) and population spike (B) following a second tetanus.

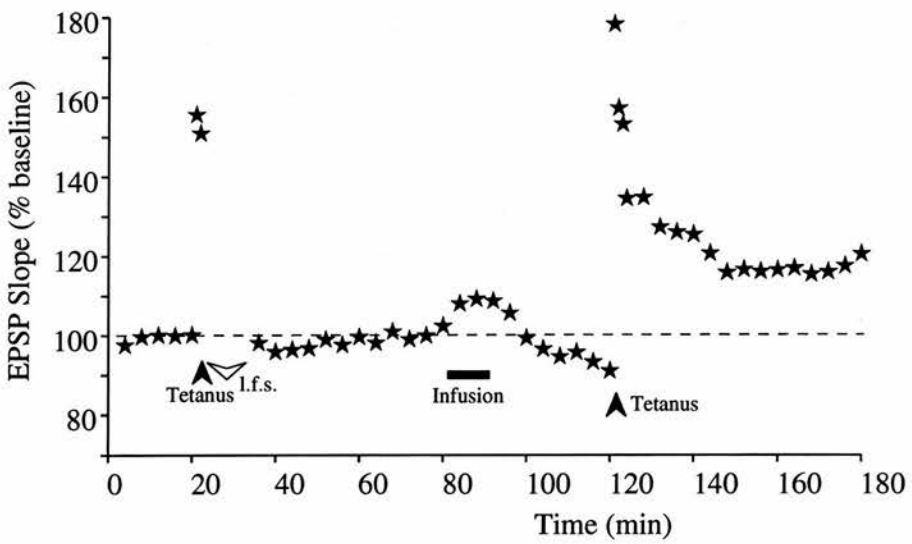


Fig 7.6.3

MCPG failed to block EPSP slope LTP even in animals showing complete depotentiation, such as the example given here.

7.7 General discussion

The experiments described in this study have consistently revealed that intraventricular infusion of a high dose of (*R,S*)-MCPG in urethane-anaesthetized rats does not result in a block of LTP in the dentate gyrus. This result holds whether LTP is assessed relative to a pre-tetanus baseline, to a concurrently stimulated control pathway, or is induced in a pathway previously subject to successful depotentiation.

In experiment 7.3, i.c.v. infusion of either 20 mM or 200 mM (*R,S*)-MCPG failed to cause a significant reduction in the level of LTP up to 2 hr after tetanization. It is unlikely that this was due to any failure of the drug infusion protocol as D-AP5 administered by the same route caused a total block of LTP.

The bilateral stimulation and recording set-up used in experiment 7.4 confirmed that the baseline changes induced by MCPG do not affect the level of potentiation 1-2 hr later (but see section 7.4.3 for qualification of this argument). It appears, however, that the baseline fall caused by MCPG is more marked after the induction of LTP followed by incomplete depotentiation (figure 7.6.1A) than the fall occurring after more effective depotentiation (figure 7.6.2A), or when MCPG is applied to an untetanized pathway (figures 7.3.11A and 7.4.1A). It is conceivable that tetanization followed by 5 Hz stimulation results in a residual mGluR-dependent potentiation which returns to baseline after MCPG application. However, MCPG has been shown to have no effect on LTP when applied after a high frequency tetanus alone (Riedel et al., 1995a). Furthermore, inspection of the data from individual rats suggests that the size of the fall in EPSP slope following MCPG infusion in experiment 7.6 was independent of the amount of LTP remaining after tetanization and 5 Hz stimulation (data not shown). This finding is inconsistent with the suggestion that the residual LTP after depotentiation is mGluR-dependent. However, the reason for the difference in baseline changes between experiments is not known.

It is unlikely that changes in brain temperature had a significant effect on the relative level of LTP recorded in experiments 7.3 and 7.4. In figure 7.3.6, the slight increase in brain temperature of approximately 0.5°C associated with MCPG infusion appeared to be transient. No significant temperature differences between groups were found 2 hr after tetanization, although the mean brain temperature of MCPG-treated rats was about 0.4°C higher at this point than in vehicle-infused controls. Nevertheless, it is unlikely that a block of LTP with 200 mM MCPG was masked by a small temperature-dependent increase in EPSP slope in this group since in experiment 7.4, vehicle-infused controls showed brain temperatures slightly (but again non-significantly) higher than MCPG-infused animals 2 hours after tetanization (data not shown), yet a block of LTP was still not obtained with MCPG.

It could be argued that the above results simply reflect an insufficient blockade of mGluR activation in the dentate gyrus. Intraventricular infusion of a drug results in an unknown distribution and concentration within the target structure; these are problems associated with most *in vivo* studies. Nevertheless, in experiment 7.5, MCPG infusion was found to inhibit the rise in spontaneous activity induced by infusion of the mGluR agonist, (1*S*,3*R*)-ACPD. Slow-onset potentiation such as that described by Manahan-Vaughan and Reymann (1995a) was not observed after ACPD infusion (see experiment 7.5). The fact that MCPG is effective in blocking an electrophysiological response to ACPD confirms that the drug reaches ACPD-activated mGlu receptors. Nevertheless, this finding is far from perfect as a positive control. For instance, it cannot conclusively be claimed that these receptors are actually in the dentate, since MCPG may be blocking a network-mediated action of ACPD originating elsewhere in the hippocampus or in other brain structures (see also experiment 7.5.4). However, an inhibition of the effects of ACPD does at least provide a crude assay for the functional blockade of mGluRs by MCPG.

It has been suggested that mGluR activation may regulate plasticity by turning on a "molecular switch" which eliminates the requirement for further mGluR activation in the induction of LTP; this switch can be reset by depotentiating stimulation (Bortolotto et al., 1994). However, in experiment 7.6, MCPG remained ineffective in blocking LTP even after effective prior depotentiation (but see experiment 7.6.4). Considering the controversy surrounding the phenomenon, it is interesting that a period of 5 Hz stimulation given 2 min after tetanization results in significant depotentiation: 10 min of this depotentiating stimulation resulted in a mean of only 5.6 ± 2.5% LTP after 1 hour, compared to 19.3 ±1.4% in tetanized-only controls. A more detailed experimental investigation of this phenomenon is presented in the following chapter.

The failure of MCPG to block LTP in the dentate gyrus of urethane-anaesthetized rats is consistent with the results of one recent study conducted under similar conditions (Bordi and Ugolini, 1995); an earlier report described a reduction in the percentage EPSP slope potentiation after acute intraventricular infusion, but no block of population spike potentiation (Richter-Levin et al., 1994). A complete block of LTP was seen only during direct perfusion of MCPG into the dentate gyrus via a push-pull cannula. However, it has been reported that urethane antagonizes the excitation induced by application of excitatory amino acid agonists, including NMDA, in isolated spinal cord (Evans and Smith, 1982). In another study, urethane anaesthesia was found to depress dentate gyrus granule cell excitability and the strength of synaptic responses (Shirasaka and Wasterlain, 1995). The recent finding that *Thy-1* knockout mice show a complete block of dentate LTP under urethane anaesthesia, but express significant LTP in the freely-moving state, demonstrates that the effects of anaesthesia cannot be ignored (Nosten-Bertrand et al., 1996; Errington et al., 1997). Consistent with this result, it is found that stronger tetanization parameters are required to induce LTP in area CA1 of urethane-anaesthetized rats, compared to the freely-moving state (Riedel et al., 1994b). It has been reported that MCPG does not block the LTP induced in area CA1 *in vitro* by strong tetanization protocols such as

theta burst stimulation (Brown and Reymann, 1995). The weaker tetanization parameters necessary in freely-moving rats may unmask a modulatory role of mGluRs, accounting for reports of a complete block of LTP by MCPG under these circumstances (see Riedel et al., 1995a).

An experiment to investigate the role of anaesthesia on the ability of MCPG to block LTP will be carried out in the near future. Rats with chronically implanted stimulating and recording electrodes will be infused with MCPG and tetanized either in the freely moving state, or under urethane anaesthesia.

However, anaesthesia may be merely one of many factors influencing studies of MCPG and LTP. Note that conflicting reports have been obtained under apparently similar conditions in the dentate gyrus of urethane-anaesthetized rats (Richter-Levin et al., 1994; Bordi and Ugolini, 1995). A drug whose effects on LTP are critically dependent on a range of experimental variables, many of them unidentified, may not be a suitable tool to probe the relationship between LTP and learning. MCPG is a very weak mGluR antagonist, with an uncertain profile of subtype selectivity (see chapter 3.6.3 & 3.10). As noted in the previous chapter, the quantity of MCPG that can be delivered is limited by solubility of the drug, together with the need for small infusion volumes *in vivo*. These problems raise questions about the suitability of MCPG for use the intact animal. In order adequately to characterize the role of mGluRs in LTP, it is likely that antagonists with considerably greater subtype specificity and potency than MCPG will be needed.

Chapter Eight

Reversal of dentate LTP

by low frequency stimulation

8.1 General introduction

Much attention has recently been devoted to the phenomena of long-term depression (LTD) and depotentiation. LTD refers to the depression of naïve pathways. Depotentiation refers to a reduction in synaptic transmission from a previously potentiated level. The mechanisms of LTD induction were discussed in chapter 2.5, and the following section will focus entirely on depotentiation.

Barrionuevo et al. (1980) found that the delivery of 1 Hz stimulation could reverse LTP in area CA1 of anaesthetized rats. A subsequent study in freely moving rats revealed that the phenomenon was input-specific (Stäubli and Lynch, 1990). A number of different laboratories have subsequently obtained depotentiation in area CA1 *in vitro* using a continuous train of 1-5 Hz stimulation (Fujii et al., 1991; Larson et al., 1993; Bashir and Collingridge, 1994; O'Dell and Kandel, 1994; Doyle et al., 1997), but others have failed to induce depotentiation under similar circumstances (Errington et al., 1995). This discrepancy is discussed later.

The mechanistic relationship between LTD and depotentiation in area CA1 is unclear. Both phenomena can be induced using the same trains of low frequency stimulation, and both are generally found to be dependent on NMDA receptor activation (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Fujii et al., 1991; Wagner and Alger, 1995), although one group has reported that depotentiation is NMDA receptor-independent, but depends on the activation of mGluRs (Bashir and Collingridge, 1994). In addition, both LTD and depotentiation are blocked by protein phosphatase inhibitors (Mulkey et al., 1993; O'Dell and Kandel, 1994). However, many studies have reported that the same stimulation parameters that induce depotentiation in the adult CA1 region do not induce LTD of naïve synapses either *in vitro* (Fujii et al., 1991; O'Dell and Kandel, 1994; Bashir and Collingridge, 1995; Otani and Connor, 1995; Wagner and Alger, 1995) or *in vivo* (Barrionuevo et al., 1980; Stäubli and Lynch, 1990; Stäubli and Scafidi, 1997; Doyle et al., 1997; but see chapter 2.5). This fact may be explained by the finding that LTD is more readily obtained in the immature rather than the adult hippocampus (Dudek and Bear, 1993; Wagner and Alger, 1995), whereas the susceptibility of CA1 synapses to depotentiation does not change in an age-dependent manner (at least in area CA1 *in vitro*; see Errington et al., 1995)

It has been suggested that depotentiation represents a form of "primed LTD" (Wexler and Stanton, 1993; Wagner and Alger, 1996). According to this model, $GABA_A$-mediated inhibition of NMDA responses is too strong in the mature hippocampus to allow the induction of LTD (see Wagner and Alger, 1995). However, tetanization results in an increase in the susceptibility for $GABA_A$ inhibition to weaken during low frequency stimulation, thus facilitating the induction of depotentiation. The successful induction of LTD in the immature hippocampus is attributed to the fact that $GABA_A$-mediated inhibition is weaker in young animals.

Much of the work on LTD and depotentiation has focussed on area CA1 and relatively few studies of dentate depotentiation have been carried out. However, heterosynaptic depotentiation is readily induced in the dentate gyrus *in vivo* (e.g. Doyère et al., 1997), and homosynaptic depotentiation has been successfully induced in slices taken from immature rats (O'Mara et al., 1995a,b). However, homosynaptic depotentiation is generally not induced by the delivery of low frequency stimulation to the medial perforant path—dentate gyrus projection of adult rats *in vivo* (Errington et al., 1995; Abraham et al., 1996; but see section 8.5 for further comments on the latter study).

In addition to the use of immature versus adult preparations, one factor that may be critical for the successful induction of depotentiation is the interval between tetanization and low frequency stimulation. A number of studies, including experiment 7.6, have reported that LTP is most readily reversed when low frequency stimulation is delivered soon after tetanization (Barrionuevo et al., 1980; Stäubli and Lynch, 1990; Larson et al., 1993). A systematic investigation of this possibility in area CA1 *in vitro* revealed that the percentage reversal of LTP declines steeply as the interval between tetanus and 5 Hz is increased. Depotentiation was found to be most effective when 5 Hz stimulation was delivered within minutes of tetanization (Stäubli and Chun, 1996).

A number of additional studies have provided evidence that LTP is vulnerable to disruption for a limited period after induction. For instance, brief cooling shocks and periods of anoxia have been reported to cause a time-dependent reversal of LTP, presumably by the interruption of biochemical cascades involved in the stabilization of LTP (Bittar and Muller, 1993; Arai et al., 1990a). Subsequent studies have implicated adenosine A_1 receptors and integrins (a class of cell surface receptor) in the time-dependent reversal of LTP (see section 8.5).

In experiment 7.6, it was reported that a 1 or 10 min period of 5 Hz stimulation results in a significant reversal of dentate LTP *in vivo* when delivered 2 min after a high frequency tetanus. The aim of the present study was to fully characterize the time-dependence of this effect. However, our interest in the possibility of reversing LTP in a time-dependent manner was motivated by more than the mere desire to characterize an electrophysiological phenomenon. The ability to selectively erase recently induced potentiation in a synapse-specific manner, if successful, may provide a powerful tool for the investigation of the relationship between LTP and learning.

8.2 The effect of the interval between tetanus and 5 Hz stimulation on the reversal of LTP

8.2.1 Methods

Adult male Lister hooded rats obtained from the departmental breeding colony were used as subjects. Animals were prepared for the recording of dentate field potentials as described in chapter 4.3. Stimulation and recording were carried out unilaterally. Owing to difficulty experienced in inducing LTP using tetanus parameters which had proved adequate throughout experiment 7, a new set of parameters was adopted (see chapter 9.4).

Stimulation consisted of monophasic pulses, the intensity of which was adjusted to elicit a population spike amplitude of 2-4 mV. The pulse width was 50 μs, except during a high frequency tetanus or 5 Hz stimulation, when the pulse-width was doubled; test pulses were delivered at 0.05 Hz throughout, except during input / output curves, when this frequency was increased to 0.1 Hz.

Figure 8.2.1 shows an outline of the experimental design used in the present study. A baseline period of variable duration (approx. 30-90 min) was initially recorded, until it was judged that evoked responses had stabilized. This was followed by an I / O curve consisting of 4 pulses at each of 10 stimulation intensities, ranging from 0.1-1.0 mA. A further baseline period of 20 min was then recorded before a high frequency tetanus was delivered. Tetanic stimulation consisted of 3 trains of 50 pulses of 100 μs duration, delivered at 250 Hz, with 10s between trains. A single test pulse of 100 μs duration was given 10s after the final tetanus train to provide an early measure of post-tetanic potentiation. After a variable interval, measured from the final tetanus train, a 10 min period of 5 Hz stimulation was delivered. The intervals tested were as follows: 10s (n = 7), 30s (n = 6), 2 min (n = 7), 10 min (n = 7), 30 min (n = 6). Control groups received either 5 Hz only, with no tetanus ("5 Hz only" group; n = 6), or tetanic stimulation only, with no subsequent 5 Hz period ("LTP control" group; n = 6). In the period between tetanus and 5 Hz, stimulation at the normal frequency and pulse width was continued, except for those animals receiving 5 Hz as early as 10 s or 30 s after tetanization, in which case no intervening stimulation was given. At the end of the 5 Hz stimulation period, test pulses were again delivered at the normal frequency and duration until 1 hr had passed since tetanization. A second I / O curve was then recorded.

8.2.2 Results

8.2.2.1 Reversal of EPSP slope potentiation

Figure 8.2.2 shows the amount of EPSP slope potentiation at increasing delays between tetanus and 5 Hz stimulation. For reference, the "5 Hz only" and "tetanus only" control groups are included in each individual graph. High-frequency tetanization alone reliably induced LTP; 5 Hz stimulation alone had little effect, although a slight increase in slope was often observed. The efficacy of LTP reversal declined as the interval between tetanus and 5 Hz increased; 5 Hz stimulation delivered 10 min or more after tetanization had little effect other than a transient depression of responses, typically lasting less than 10 min.

The mean percentage LTP in each group, measured over the final 10 min of the experiment (50-60 min after tetanization), is plotted in figure 8.2.3. An ANOVA revealed a highly significant overall effect of group [$F(6,38) = 10.2$; $p < 0.0001$]. A significant reversal of LTP was only seen when less than 10 min intervened between tetanus and 5 Hz [*post-hoc* Newman-Keuls pairwise comparisons of the amount of LTP in the "tetanus only" control group versus LTP in all other groups; see figure legend]. Delivery of 5 Hz starting 10s after tetanization resulted in a complete reversal of LTP. No significant difference was found between the slight increase in EPSP slope seen with 5 Hz alone, and that seen in the "10s" group. In fact, only the "10 min", "30 min" and "tetanus only" groups were found to exhibit significant LTP relative to the "5 Hz only" condition [$p < 0.01$ in each case; Newman-Keuls].

No significant group differences were found in absolute mean EPSP slope values over the 10 min before tetanization (table 8.2.1). A measure of initial slope "PTP" was calculated for all tetanized groups by dividing the value recorded 10 s after the final tetanus train by the value obtained from the first pulse of the first tetanus train, then multiplying by 100. No group difference was found in this measure of early slope PTP [figure 8.2.5A; $F < 1$], indicating that the initial potentiation induced by tetanization was identical across groups (excluding the "5 Hz only" condition).

8.2.2.2 Reversal of population spike potentiation

The population spike data were generally consistent with the EPSP slope data. Figure 8.2.5 shows the absolute increase in population spike amplitude at all intervals between tetanus and 5 Hz. "Tetanus only" and "5 Hz only" control groups are again included in each individual graph. High-frequency tetanization alone induced a lasting increase in population spike amplitude. However, 5 Hz stimulation alone caused a small but lasting depression of the population spike.

The mean population spike increase over the final 10 min of the experiment is shown in figure 8.2.6.

The "5 Hz only", "10s" and "30s" groups showed significantly less LTP than "tetanus only" controls (see figure legend). Delivery of 5 Hz stimulation 2 min or more after tetanization caused only a transient depression in population spike amplitude, which although outlasting the EPSP slope depression, typically recovered within about 20 min.

No significant group differences were found in absolute baseline population spike amplitude over the 10 min prior to tetanization; the test pulse stimulation intensities required to elicit population spikes of this amplitude did not differ across groups (table 8.2.1). An analysis of population spike "PTP" recorded 10s after tetanization revealed no group differences in initial potentiation [figure 8.2.4B; $F(5,33) = 1.41$; $p > 0.2$].

Table 8.2.1 Baseline parameters

	Stimulation Intensity (µA)	**Initial EPSP slope (mV / ms)**	**Initial pop. spike amplitude (mV)**
5 Hz only	286.7 ± 33.1	4.63 ± 0.26	3.09 ± 0.33
10 s	287.1 ± 14.8	4.55 ± 0.23	3.17 ± 0.52
30 s	298.3 ± 36.8	5.10 ± 0.38	3.19 ± 0.72
2 min	297.1 ± 46.4	4.49 ± 0.32	2.85 ± 0.43
10 min	275.7 ± 30.6	4.69 ± 0.22	2.14 ± 0.15
30 min	286.7 ± 25.8	4.90 ± 0.24	2.91 ± 0.22
Tetanus only	280.0 ± 43.9	4.34 ± 0.36	2.54 ± 0.50
ANOVA results	$F < 1$	$F < 1$	$F < 1$

8.2.2.3 Changes in EPSP / spike (E-S) coupling induced by 5 Hz stimulation

Mean EPSP slope and population spike amplitude values were calculated for each stimulation intensity sampled during both the initial and final I / O curves, i.e. 20 min before and 60 min after tetanization. The maximum mean EPSP slope and population spike amplitude recorded during the initial I / O curve were arbitrarily assigned values of 100 %, to which all other values were normalized. An example of such I / O curves recorded from an animal in the tetanus only group is shown in figure 8.2.7. In this example, EPSP slope and population spike LTP was observed over a broad range of sampling intensities.

For each rat, scatter plots relating EPSP slope to population spike amplitude were constructed from the I / O curve data obtained both before and after tetanization and 5 Hz stimulation (or before and after 5 Hz alone in the "5 Hz only" group). Figure 8.2.8 shows sample scatter plots constructed from the I / O curves displayed in figure 8.2.7. Data points at which the population spike amplitude was zero were excluded. Linear regression lines have been fitted to the scatter plots before and after tetanization. After tetanization, data points exhibited an overall shift in a direction parallel to the pre-tetanus regression line. This shift indicates that both population spike and EPSP slope LTP were induced. In the absence of any changes in E-S coupling, regression lines should fall directly on top of each other before and after tetanization, despite the shift along the regression line mentioned above. However, the slight leftward shift in figure 8.2.8 indicates that population spike amplitude was potentiated to a slightly greater extent than that predicted by the EPSP slope increase, i.e. modest E-S potentiation occurred.

Figure 8.2.9 shows mean E-S plots for each group. For display purposes, data points have been connected by interpolation. As indicated by the slight mean leftward shift in the E-S relationship, LTP in "tetanus only" controls was accompanied by a slight E-S potentiation, consistent with the example shown in figure 8.2.8. However, 5 Hz stimulation alone caused a rightward shift in the E-S relationship, indicating a decrease in E-S coupling. Little change in E-S coupling was seen in any of the groups that received a tetanus followed by 5 Hz stimulation (see below).

In order to analyse these data quantitatively, linear regression lines were fitted to the scatter plots obtained from each individual animal, as in figure 8.2.8. All correlation coefficients were highly significant with p values never exceeding 0.01 (data not shown). As illustrated in figure 8.2.8, the spike amplitude elicited by an EPSP slope 75 % of the maximum value recorded during the pre-tetanus I / O curve was interpolated from each regression equation. This value was compared before and after tetanization (or before and after 5 Hz stimulation alone). A significant group by time point interaction was found, revealing that groups showed differential changes in E-S coupling between the initial and final I / O curves [$F(6,38) = 3.96$; $p < 0.01$; within-subject ANOVA]. Subsequent analysis of simple effects revealed no group differences in the spike amplitude elicited by a 75 % maximal EPSP slope in the pre-tetanus I / O curve [$F < 1$], but the group difference was highly significant in the final I / O curve [$F(6,52) = 3.25$; $p < 0.01$]. Significant E-S potentiation was found in the "tetanus only" group [$F(1,38) = 6.89$; $p < 0.05$], and significant E-S depression in the "5 Hz only" group [$F(1,38) = 11.9$; $p < 0.01$]. No significant changes in E-S coupling were obtained in any group receiving a tetanus followed by 5 Hz stimulation [10 s: $F < 1$; 30 s: $F < 1$; 2 min: $F < 1$; 10 min: $F < 1$; 30 min: $F(1,38) = 2.92$; $p > 0.05$].

Figure 8.2.10 shows the mean percentage increase or decrease in population spike amplitude elicited by a 75 % maximal EPSP slope in the final I / O curve, compared to the value recorded in the initial I / O curve. In this way, the changes in E-S coupling analysed in the above ANOVA are represented by

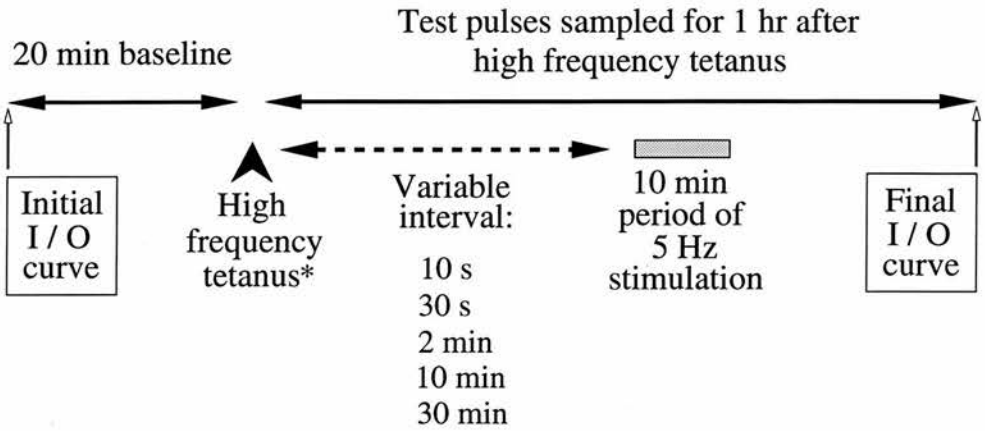
a single value. Positive values represent an increase in population spike in excess of that predicted by the increase in EPSP slope, i.e. E-S potentiation. Negative values indicate a decrease in E-S coupling. As revealed in the ANOVA, E-S potentiation and E-S depression were induced by tetanization and 5 Hz stimulation respectively. The non-significant trend towards E-S depression in the "30 min group" was due to the fact that 5 Hz stimulation finished only 20 min before the second I / O curve in this group. At this time point, population spike amplitude had not recovered to pre-5 Hz levels, resulting in an apparent slight E-S depression.

8.2.2.4 Epileptiform afterdischarges induced by 5 Hz stimulation

5 Hz stimulation was always accompanied by epileptiform activity characterized by seizure-like afterdischarges and multiple population spikes. Fig. 8.1.11 shows a continuous polygraph record during 5 Hz stimulation. The large negative deflections are positive-going EPSPs. In this example, afterdischarges began within 10 s of the start of 5 Hz stimulation and persisted for approximately 1.5 min, after which no further episodes of afterdischarge activity were observed. Representative sample EPSPs at three different time points are illustrated.

The afterdischarge onset latency and total duration were measured for each rat. Analysis of data from all rats revealed that these two indices of the severity of afterdischarge activity were negatively correlated, i.e. the more rapidly afterdischarges began, the longer they persisted [figure 8.2.12; $r = -0.41$; $p < 0.05$]. However, no significant group difference was found in afterdischarge onset latency [figure 8.2.13A; $F(5,33) = 2.17$; $p > 0.05$] or duration [figure 8.2.13B; $F < 1$], indicating that seizure-like activity was equally severe regardless of the interval between tetanus and 5 Hz, or indeed regardless of whether a tetanus was delivered at all.

Similarly, there was no overall correlation between afterdischarge onset latency and the level of LTP 1 hr after tetanization [EPSP slope LTP: $r = -0.14$, NS; population spike LTP: $r = -0.26$, NS; data not shown]. No correlation was found between afterdischarge duration LTP 1 hr after tetanization [EPSP slope LTP: $r = -0.05$, NS; population spike LTP: $r = -0.08$, NS; data not shown]. These findings indicate that the time window during which LTP is vulnerable to reversal cannot be explained as an artifact of the severity of afterdischarges elicited by 5 Hz stimulation.



* 3 trains of 50 pulses at 250 Hz;
' 2 x test pulse width

Fig. 8.2.1

Summary of experimental design. 5 Hz stimulation was delivered at intervals ranging from 10 s to 30 min following a high frequency tetanus.

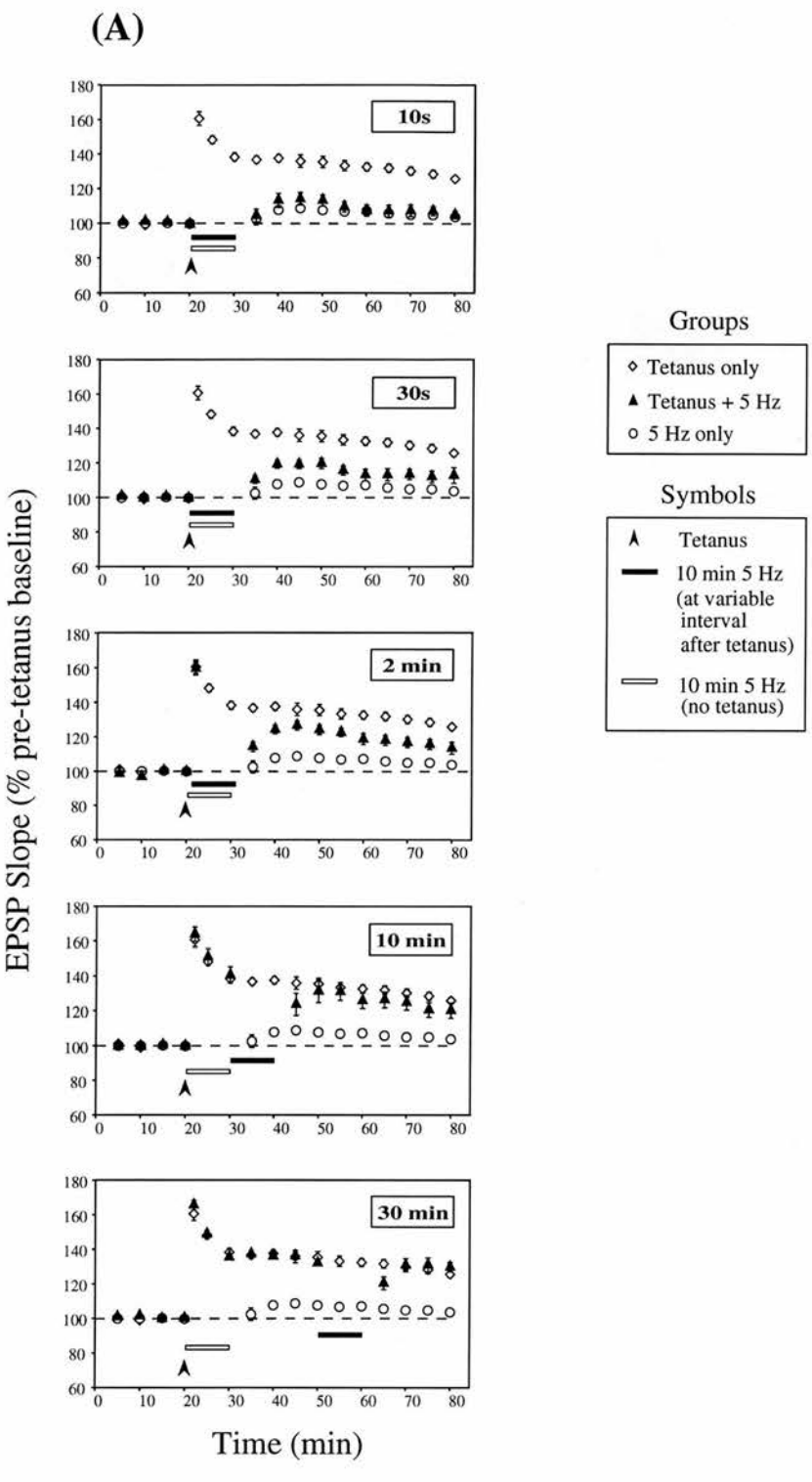


Fig. 8.2.2

EPSP slope values normalized to the pre-tetanus baseline at increasing delays between tetanization and 5 Hz stimulation. For reference, "tetanus only" and "5 Hz only" groups are included in each individual panel.

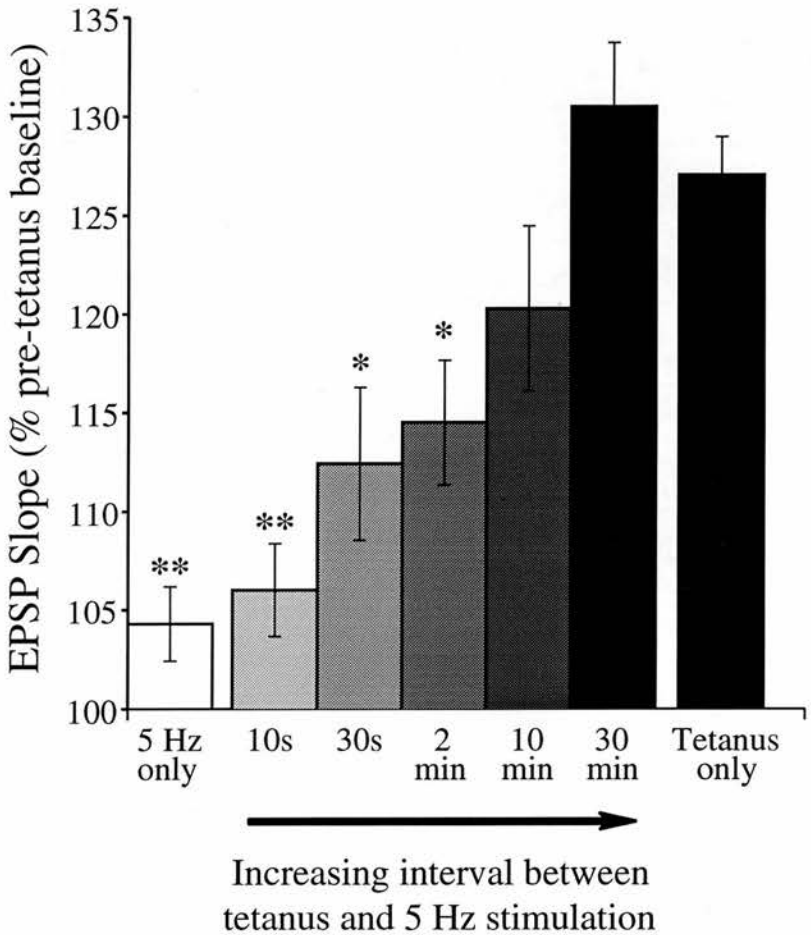


Fig. 8.2.3

Mean EPSP slope values 50-60 min after tetanization. Asterisks indicate groups showing significantly less LTP than "tetanus only" controls (* $p < 0.05$; **$p < 0.01$).

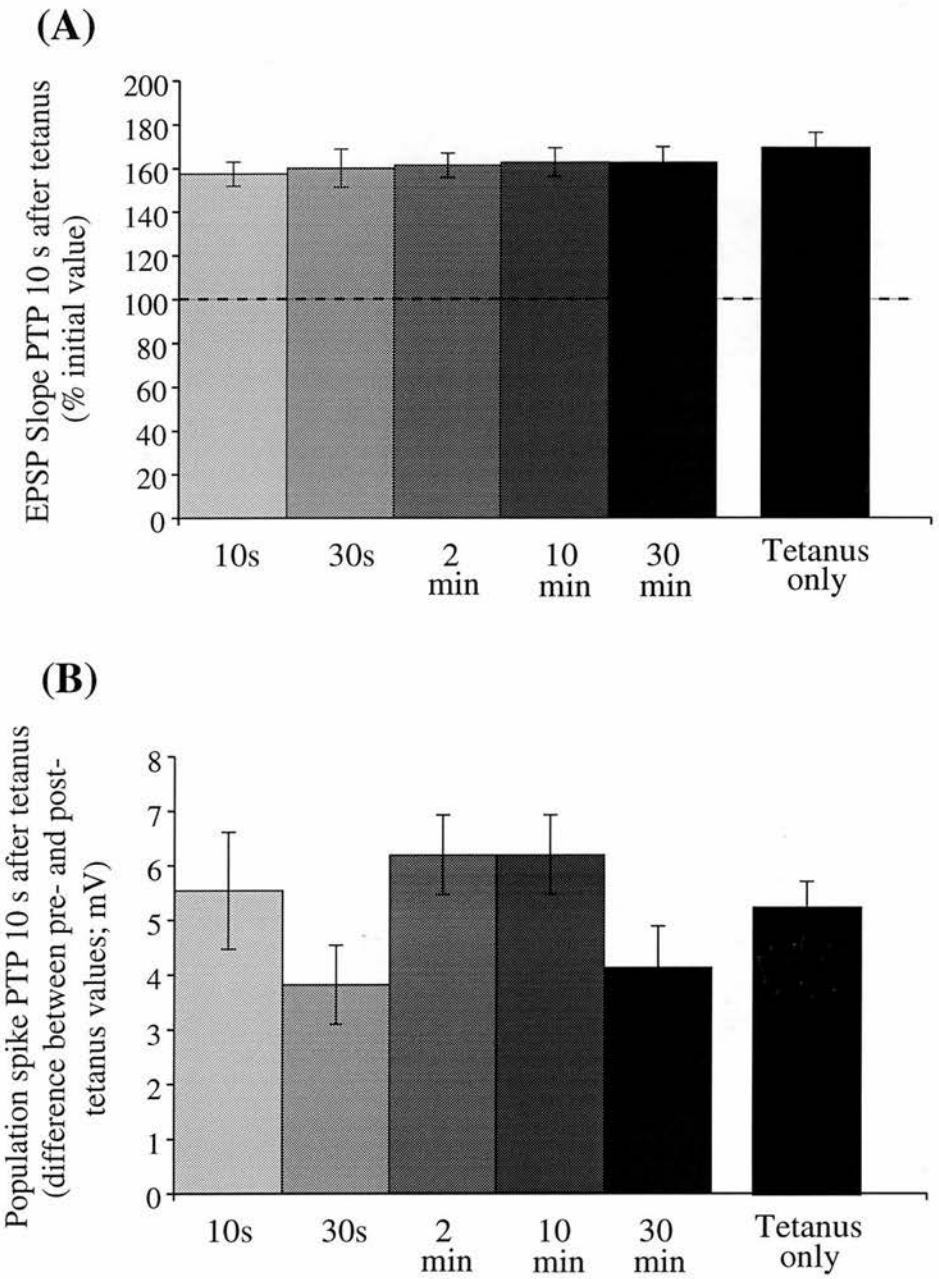


Fig. 8.2.4

PTP recorded exhibited by a single test pulse delivered 10 s after the final tetanus train. Data were normalized to the value recorded in response to the first pulse of the first tetanus train (i.e. before potentiation was induced). No group differences in either EPSP slope or population spike PTP were found.

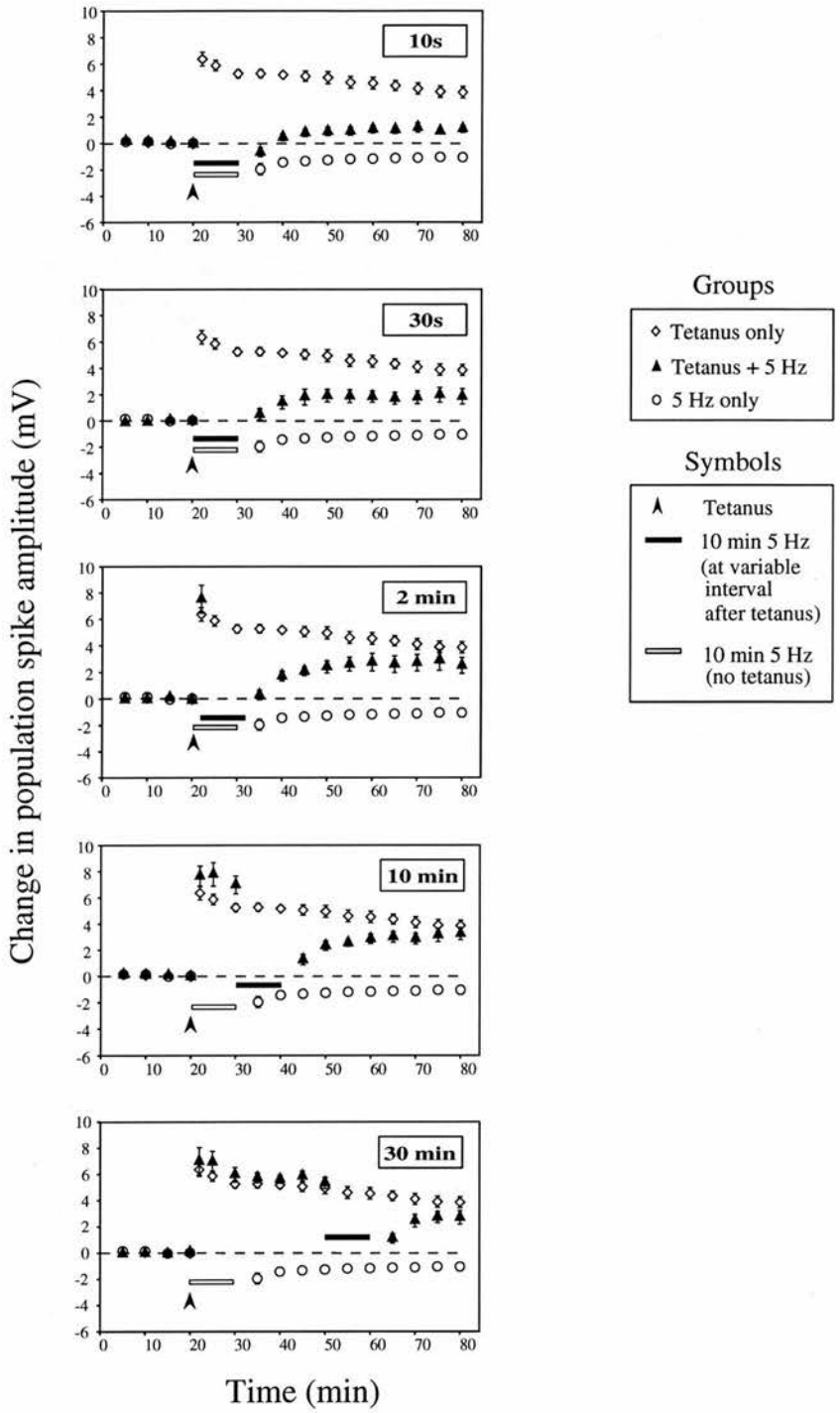


Fig. 8.2.5

Changes in population spike amplitude normalized to the pre-tetanus baseline at increasing delays between tetanization and 5 Hz stimulation. For reference, "tetanus only" and "5 Hz only" groups are included in each individual panel.

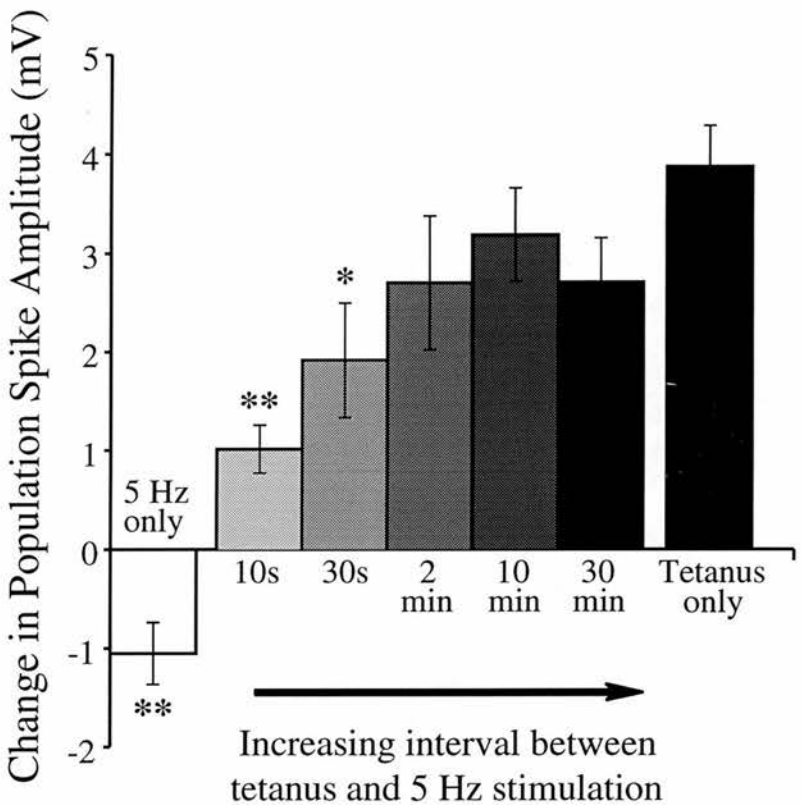


Fig. 8.2.6

Mean increase in population spike amplitude 50-60 min after tetanization. Asterisks indicate groups showing significantly less LTP than "tetanus only" controls (* $p < 0.05$; **$p < 0.01$).

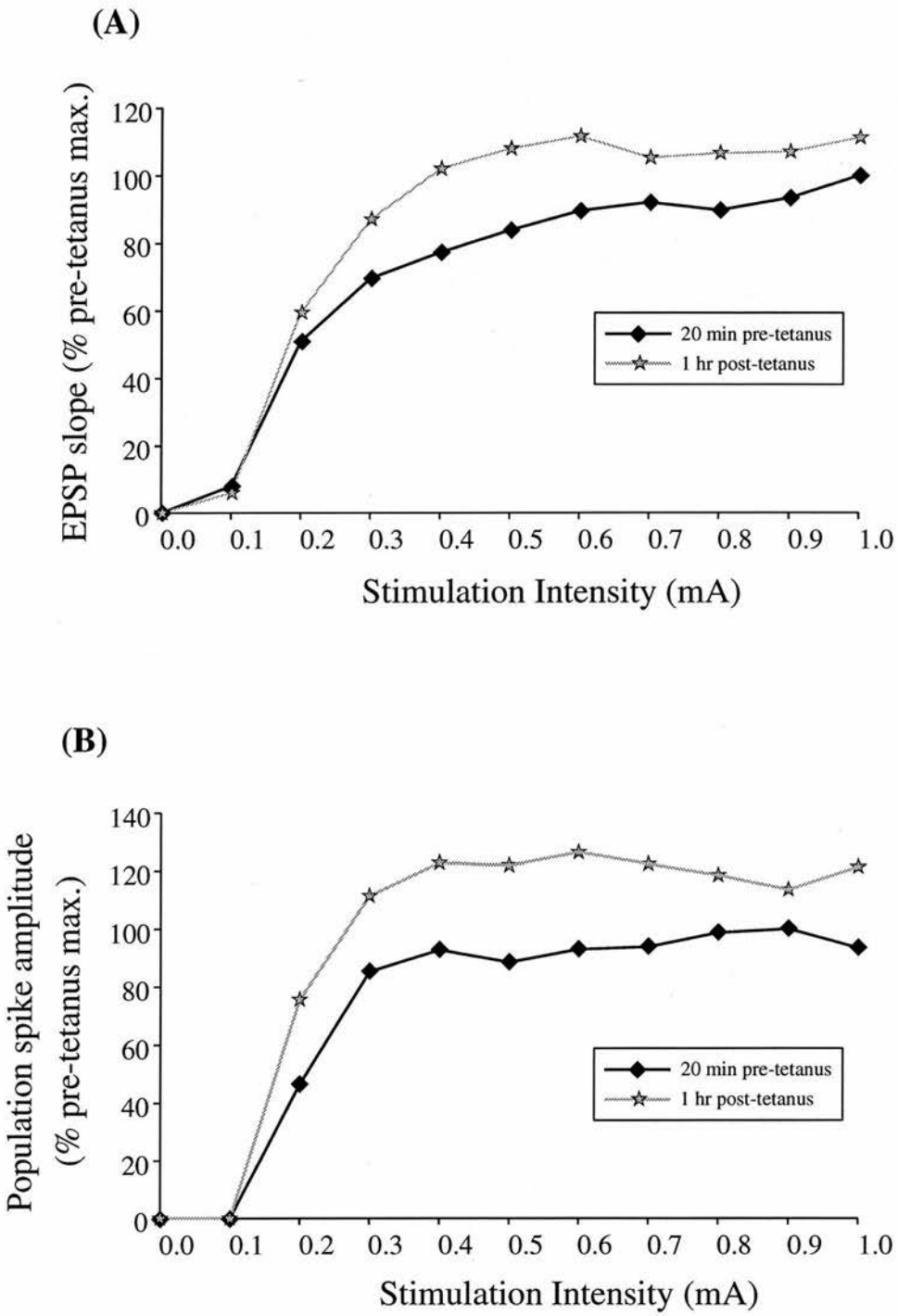


Fig. 8.2.7

Input / output curves recorded before and after tetanization in a rat receiving high frequency tetanization only. Both EPSP slope (A) and population spike potention (B) are plotted.

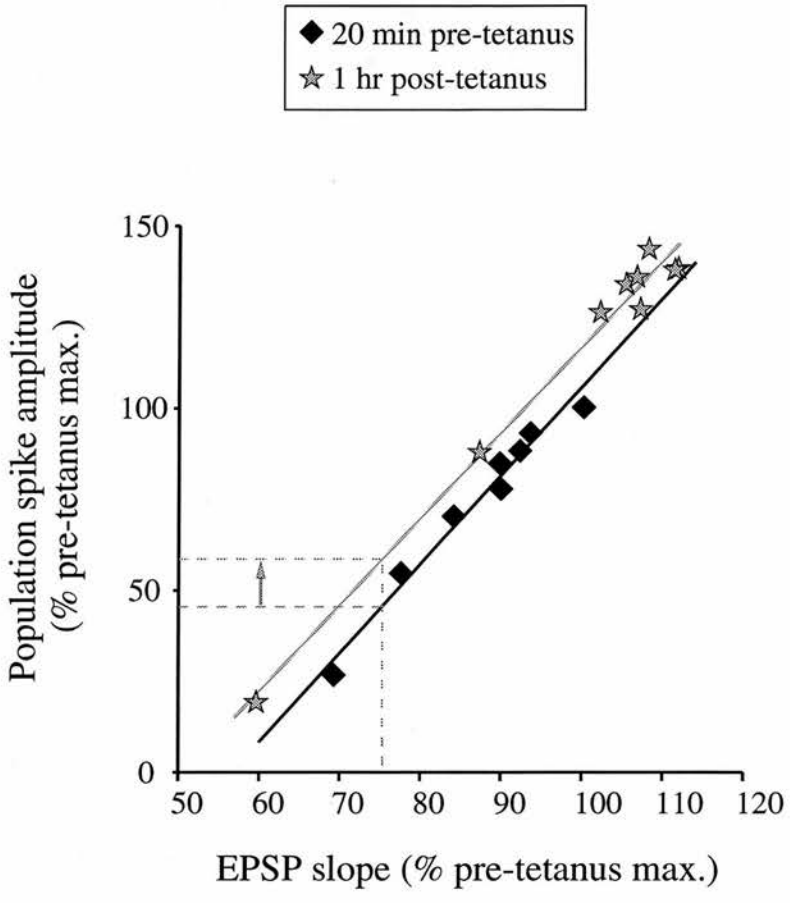


Fig. 8.2.8

Scatter plots relating population spike amplitude to EPSP slope 20 min before and 1 hr after tetanization in a single rat from the "tetanus only" group. The grey arrow and dotted lines indicate the increase in population spike elicited by a 75 % maximal EPSP slope after tetanization. E-S potentiation was modest in this example.

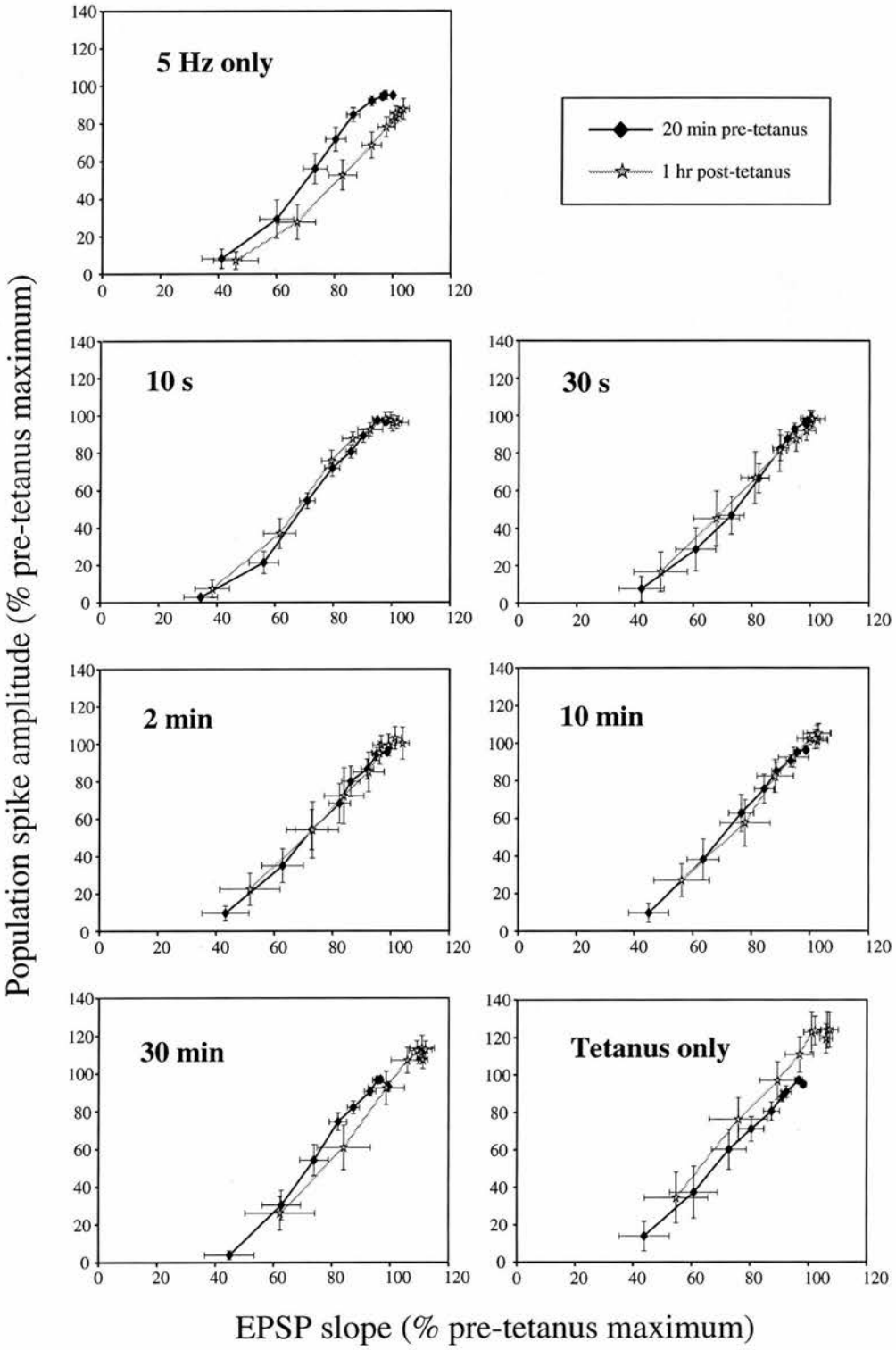


Fig. 8.2.9

Mean relationship between population spike amplitude and EPSP slope at each point on the I / O curve recorded before and after tetanization. Data from all groups are plotted (see text for details).

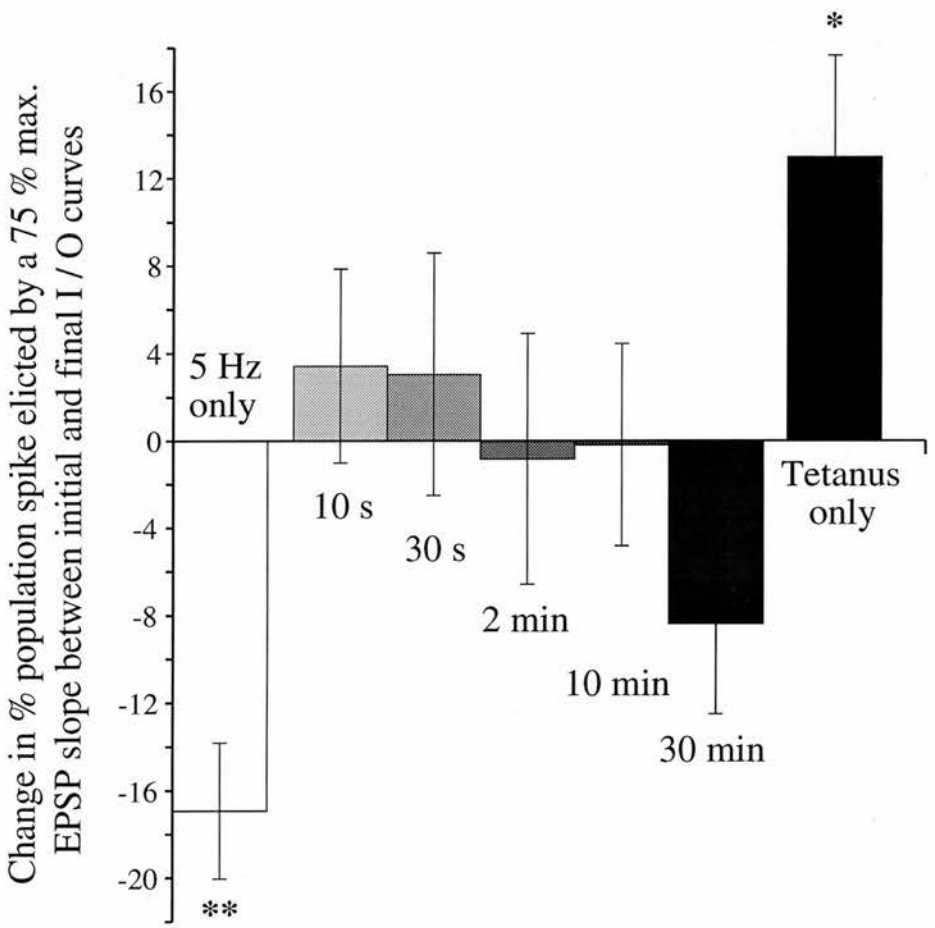


Fig. 8.2.10

Changes in population spike amplitude elicited by a fixed EPSP slope before and after tetanization and / or 5 Hz stimulation. Asterisks indicate groups in which a significant change in E-S coupling was observed.

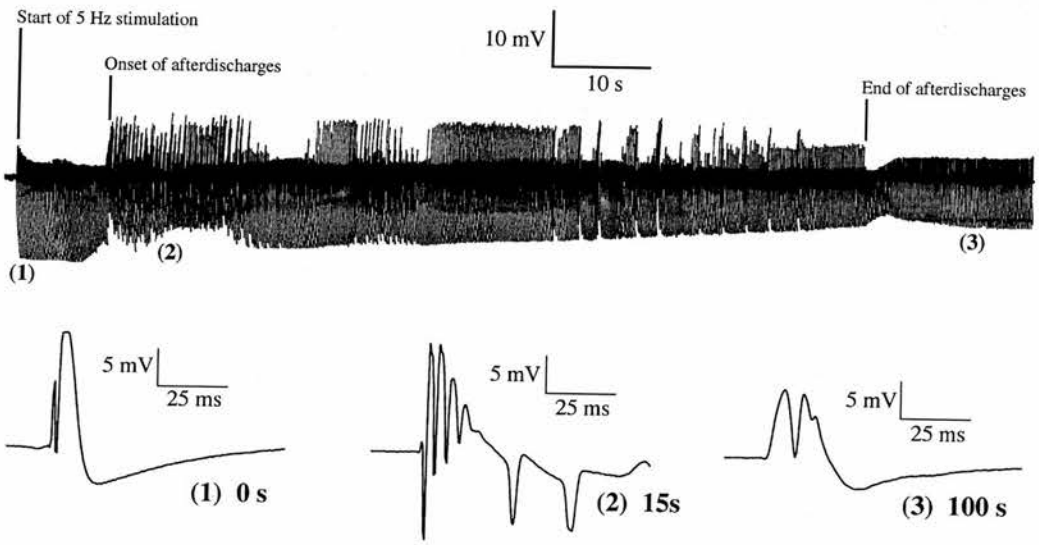


Fig. 8.2.11

Sample polygraph record during 5 Hz stimulation. The large negative deflections represent positive-going EPSPs. Epileptiform afterdischarges began abruptly within 10 s of the start of 5 Hz stimulation, and continued for approximately 1.5 min before subsiding. Representative EPSPs recorded at 0, 15 and 100 s after the start of 5 Hz stimulation are illustrated. The waveform recorded at 15 s displays the multiple population spikes characteristic of afterdischarge activity.

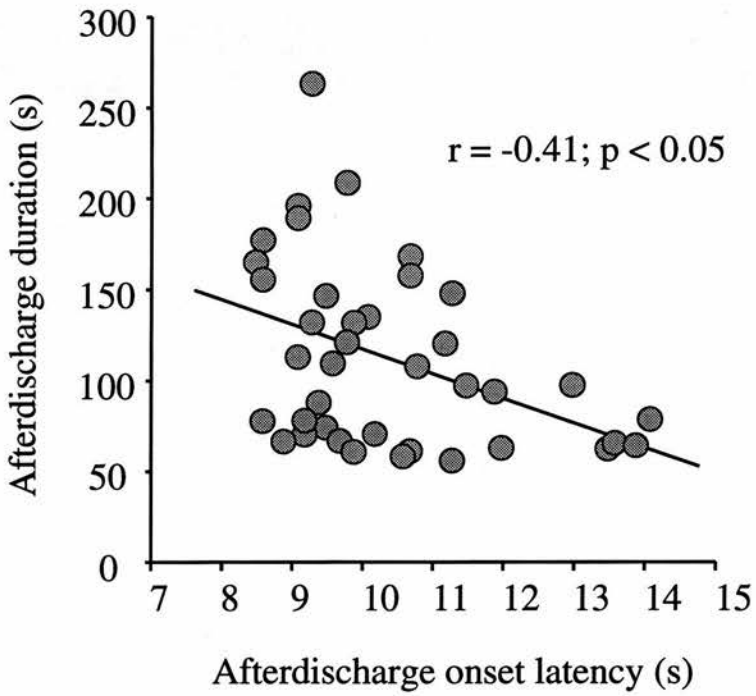


Fig. 8.2.12

Afterdischarge duration was negatively correlated with afterdischarge onset latency.

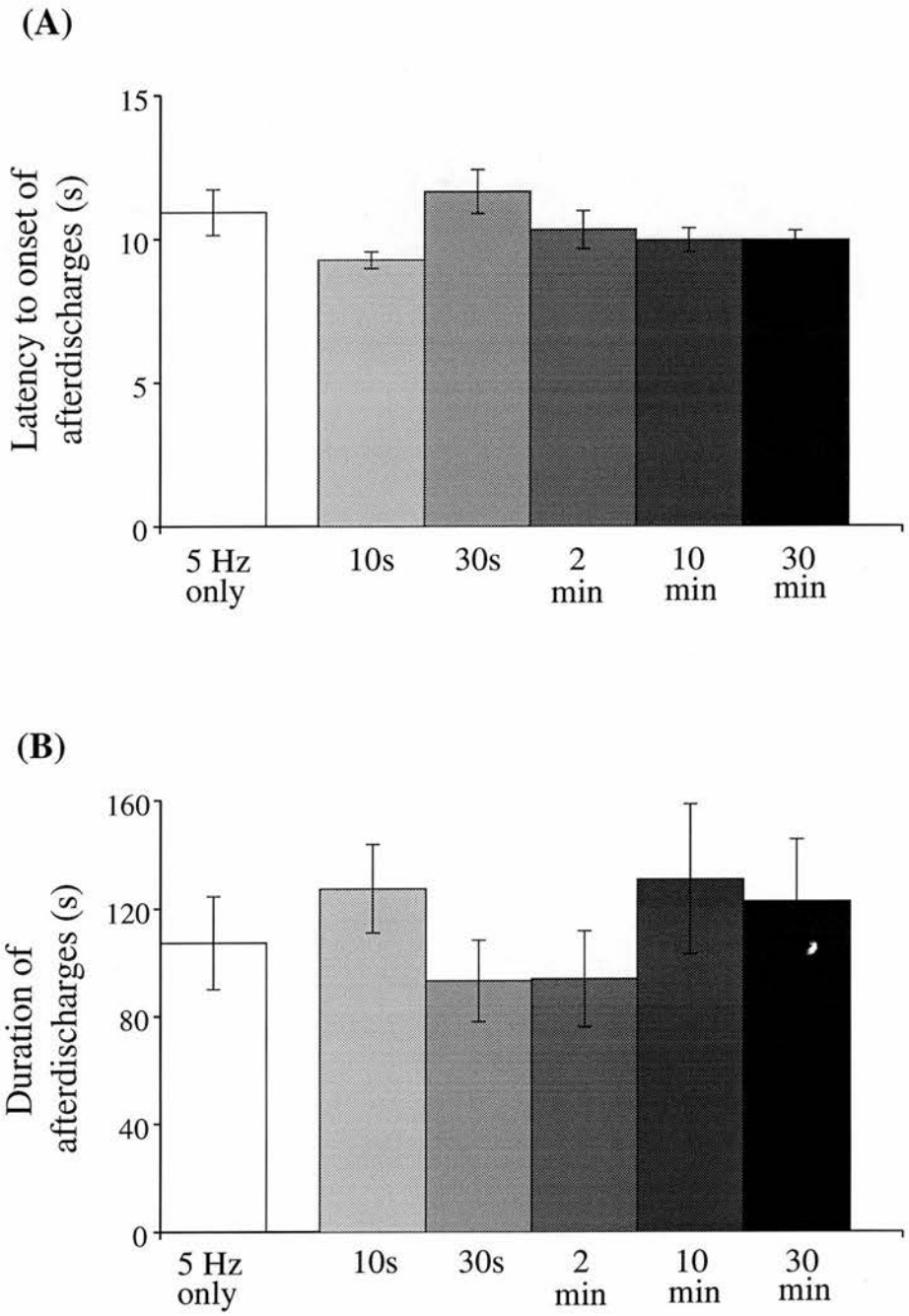


Fig. 8.2.13

The latency to onset (A) and duration of afterdischarges (B) did not differ across groups.

8.3 The effect of (*R*,*S*)-MCPG on the reversal of LTP by 5 Hz stimulation

8.3.1 Methods

Rats received intraventricular infusions of 200 mM (*R*,*S*)-MCPG (n = 3) prior to tetanization only (n = 3) or tetanization followed 10 s later by 10 min of 5 Hz stimulation (n = 3). Intervals other than 10 s were not tested because this was found to be the optimal interval for reversing LTP in experiment 8.2.

Drug solutions were prepared and infusions were carried out as described in experiment 7.2.2. Drugs were infused over 10 min, finishing 30 min before tetanization. All other aspects of the protocol were identical to that described in experiment 8.2. Owing to the small group sizes, a statistical analysis of the following data was not carried out.

8.3.2 Results

8.3.2.1 EPSP slope and population spike LTP

Figure 8.3A shows the EPSP slope data for this experiment, normalized to the pre-infusion baseline owing to the slight fall in baseline induced by MCPG infusion (see chapter 7). Consistent with the results presented in chapter 7, (*R*,*S*)-MCPG did not block LTP induced by tetanization 30 min after the end of infusion. The reversal of LTP with 5 Hz stimulation delivered 10 s after tetanization was also unaffected. Similar levels of EPSP slope PTP 10 s after tetanization were recorded in both groups (table 8.3). Population spike LTP data followed a similar pattern to the EPSP slope results (figure 8.3B). However, these data are less clear owing to the relatively poor LTP and large standard errors in the tetanus only group.

8.3.2.2 Comparison with experiment 8.2

Table 8.3 shows the EPSP slope LTP and population spike LTP obtained in the present experiment, compared to data obtained in the "tetanus only" and "10 s" groups in experiment 8.2, in which no drug infusions were given. LTP data were all measured over the final 10 min of the experiment, 50-60 min after tetanization. The level of slope PTP measured 10 s after the final tetanus train is also compared. All measures of potentiation in both tetanus only and tetanus + 5 Hz conditions were broadly similar in the presence or absence of MCPG.

Note, however, that whereas data from experiment 8.2 are normalized to the pre-tetanus baseline,

data from the present experiment are normalized to the pre-infusion baseline, on the assumption that the MCPG-induced baseline fall is transient. However, this assumption may not be entirely valid (see chapter 7.4.3), a possibility which might explain the apparent slight reduction in all measures of LTP in the presence of MCPG.

Table 8.3 A comparison of the amount of LTP and depotentiation obtained in the present study after MCPG infusion, with data from equivalent groups in experiment 8.2 in the absence of drug infusion.

		EPSP slope LTP (% pre-infusion baseline)	**EPSP slope "PTP", 10 s after tetanus (% baseline; see experiment 8.2)**	**Increase in population spike amplitude (mV)**
Tetanus only	**No drug**	127.1 ± 1.9	169.5 ± 6.8	3.87 ± 0.42
	MCPG	122.7 ± 2.7	152.0 ± 5.5	2.32 ± 1.10
Tetanus + 5 Hz	**No drug**	106.0 ± 2.4	157.5 ± 5.5	1.01 ± 0.24
	MCPG	104.6 ± 1.5	154.8 ± 7.7	0.78 ± 0.51

The values of baseline parameters (stimulation intensity, baseline EPSP slope, and baseline population spike amplitude) were similar in the present experiment to those recorded in experiment 8.2 (data not shown).

8.3.2.3 MCPG and afterdischarge activity

MCPG has been reported to have anticonvulsant properties (Tebano et al., 1997; Burke and Hablitz, 1995; Arvanov et al., 1995). Hence it might be expected to inhibit the occurrence of afterdischarges during 5 Hz stimulation. However, afterdischarges were always observed in the presence of MCPG, and onset latency and duration were within the same range as the values obtained in experiment 8.2 in the absence of the drug (data not shown).

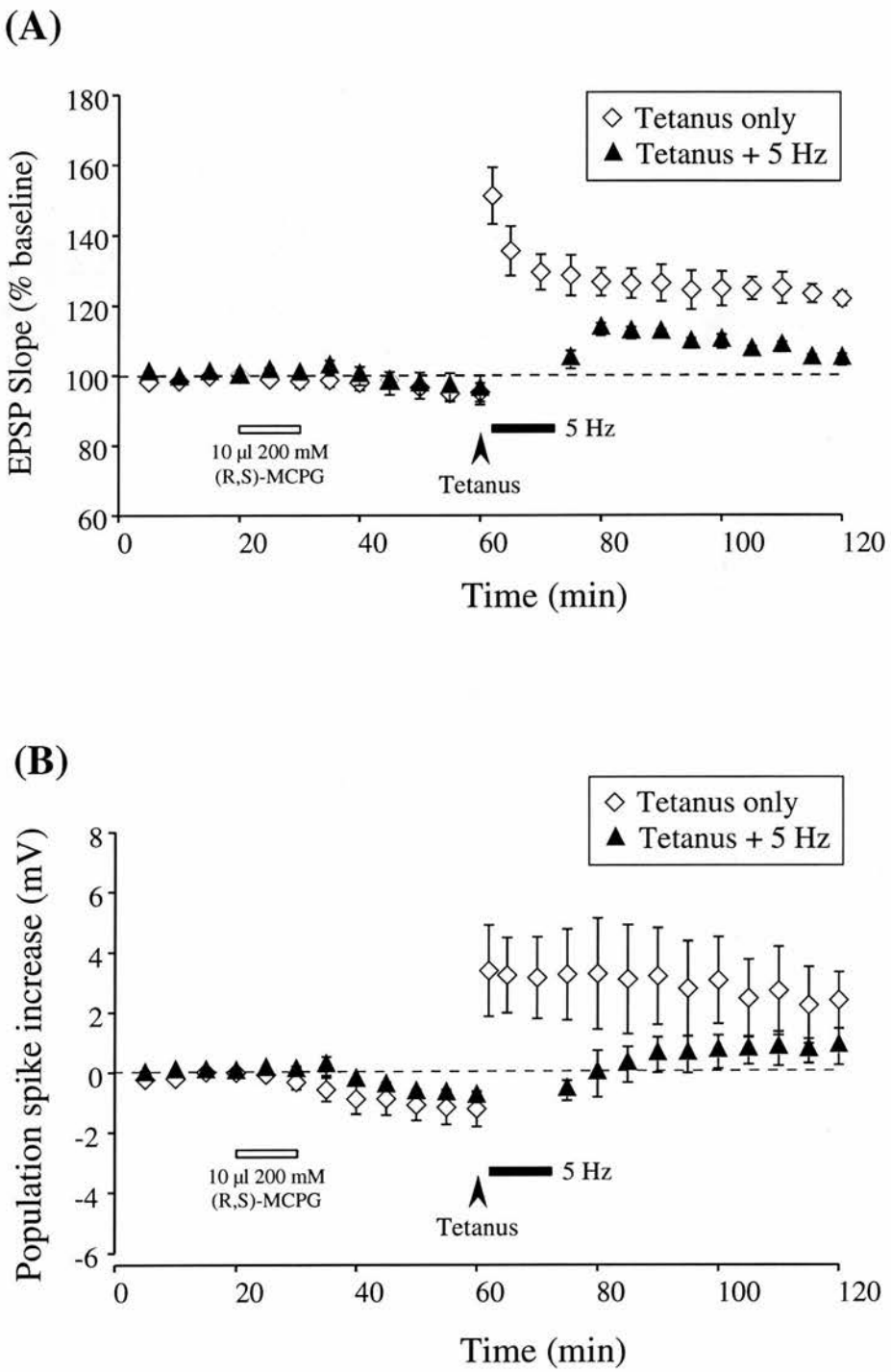


Fig. 8.3

(A) MCPG infusion failed to prevent the reversal of EPSP slope (A), or population spike LTP (B) by 5 Hz stimulation starting 10 s after tetanization.

8.4 The effect of 1 Hz stimulation on LTP

8.4.1 Methods

The methods used were identical to those described in experiment 8.2, except that a 10 min period of 1 Hz stimulation was delivered instead of 5 Hz stimulation. Because a 10 s interval between tetanus and 5 Hz stimulation was most effective in reversing LTP in experiment 8.2, this interval alone was tested in the present experiment (n = 6). Owing to the fact that this study was carried out several months after experiments 8.2 and 8.3, a new control group was deemed necessary. Hence, an additional group of rats received a tetanus only (n = 6).

8.4.2 Results

8.4.2.1 Effect of 1 Hz stimulation on LTP

The delivery of a 10 min period of 1 Hz stimulation starting 10s after tetanization had no lasting effect on EPSP slope LTP (figure 8.4.1A), although a transient depression lasting approximately 20 min was observed. The mean percentage slope LTP over the final 10 min of the experiment failed to reveal a difference between tetanus + 1 Hz and tetanus only groups [$F < 1$]. No difference in mean population spike LTP was found over the same period [figure 8.4.1B; $F(1,10) = 1.48$; $p > 0.2$].

No group differences in EPSP slope PTP measured 10 s after the final tetanus train were found [$F < 1$; data not shown]. Stimulation intensity, baseline EPSP slope, and baseline population spike amplitude were also equivalent in both groups [$F < 1$ in all cases; data not shown].

Despite the absence of a difference between 1 Hz + tetanus and tetanus only groups, the amount of EPSP slope LTP in the latter group was significantly lower than that obtained under apparently identical conditions in the tetanus only group of experiment 8.2 [115.3 ± 2.1 % and 127.0 ± 1.9 % respectively; $F(1,10) = 18.8$; $p < 0.01$]. However, no differences in population spike LTP [$F < 1$], or EPSP slope PTP 10 s after the final tetanus train [$F(1,10) = 1.30$; $p > 0.2$] were found between the two tetanus only control groups. The groups also did not differ significantly in terms of baseline EPSP slope [$F < 1$], baseline population spike amplitude [$F < 1$], and test pulse stimulation intensity [$F(1,10) = 2.07$; $p > 0.1$]. The reason for this difference in control LTP between experiments remains unknown (but see chapter 9.4.6).

8.4.2.2 Effect of 1 Hz stimulation on E-S coupling

Changes in E-S coupling were analyses as described in experiment 8.2. Figure 8.4.2A shows mean E-S plots for the tetanus only group. A very slight E-S potentiation was observed. Little change in E-S coupling was obtained after tetanization followed by 1 Hz stimulation (figure 8.4.2B).

Linear regression lines were fitted to scatter plots relating population spike amplitude to EPSP slope before and after tetanization, as described in experiment 8.2. All correlation coefficients were highly significant ($p < 0.01$ or less). An ANOVA of the population spike amplitude elicited by a 75 % maximal EPSP slope before and after tetanization and / or 5 Hz stimulation revealed a significant group by time point interaction [$F(1,10) = 3.50$; $p < 0.05$], demonstrating that the two groups showed differential changes in E-S coupling between the initial and final I / O curves. However, an analysis of simple effects failed to reveal significant E-S shifts in either group when analysed individually [tetanus only: $F(1,10) = 3.24$; $p > 0.1$; tetanus + 5 Hz: $F(1,10) = 2.09$; $p > 0.1$]. Despite this, a significant group difference in population spike amplitude elicited by a 75 % EPSP slope was found after tetanization [$F(1,14) = 7.08$; $p < 0.05$], but not before [$F < 1$]. These data provide weak evidence that whilst 1 Hz stimulation does not reverse LTP, it may reverse E-S potentiation. However, the fact that E-S potentiation did not reach significance in the tetanus only group means that there is no adequate baseline against which a reversal of E-S potentiation can be judged. Owing to the fact that 1 Hz stimulation was always delivered after a tetanus, the effects of 1 Hz alone on E-S coupling are unknown.

8.4.2.3 Absence of afterdischarges during 1 Hz stimulation.

Afterdischarges were never observed during the delivery of 1 Hz stimulation, although a slight depression of EPSPs was observed during the low frequency train (data not shown). Slope values typically fell to approximately 90 % of baseline during this period.

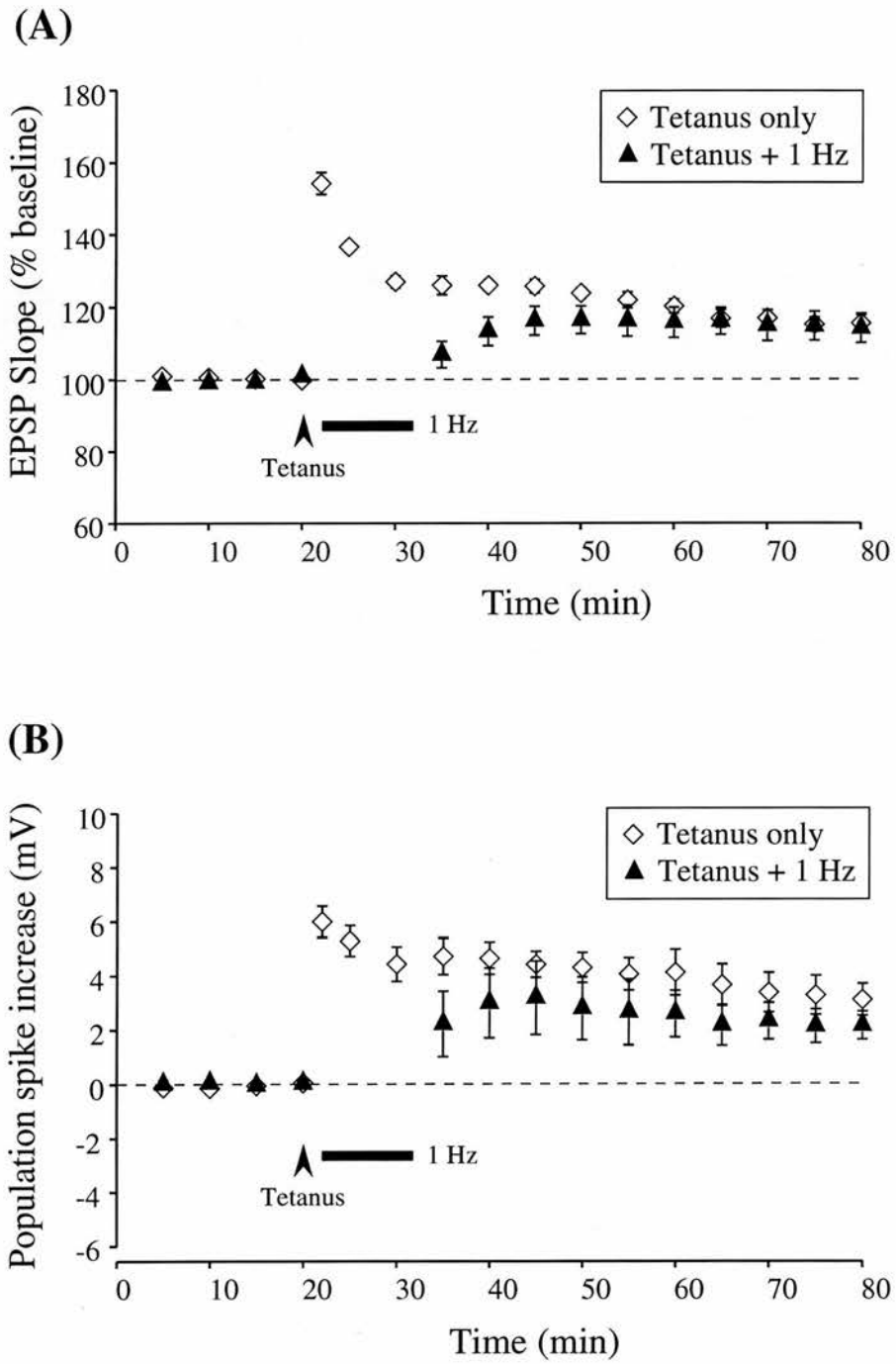


Fig. 8.4.1

(A) A 10 min period of 1 Hz stimulation did not reverse EPSP slope LTP when delivered starting 10 s after tetanization. (B) The population spike increase was also unaffected.

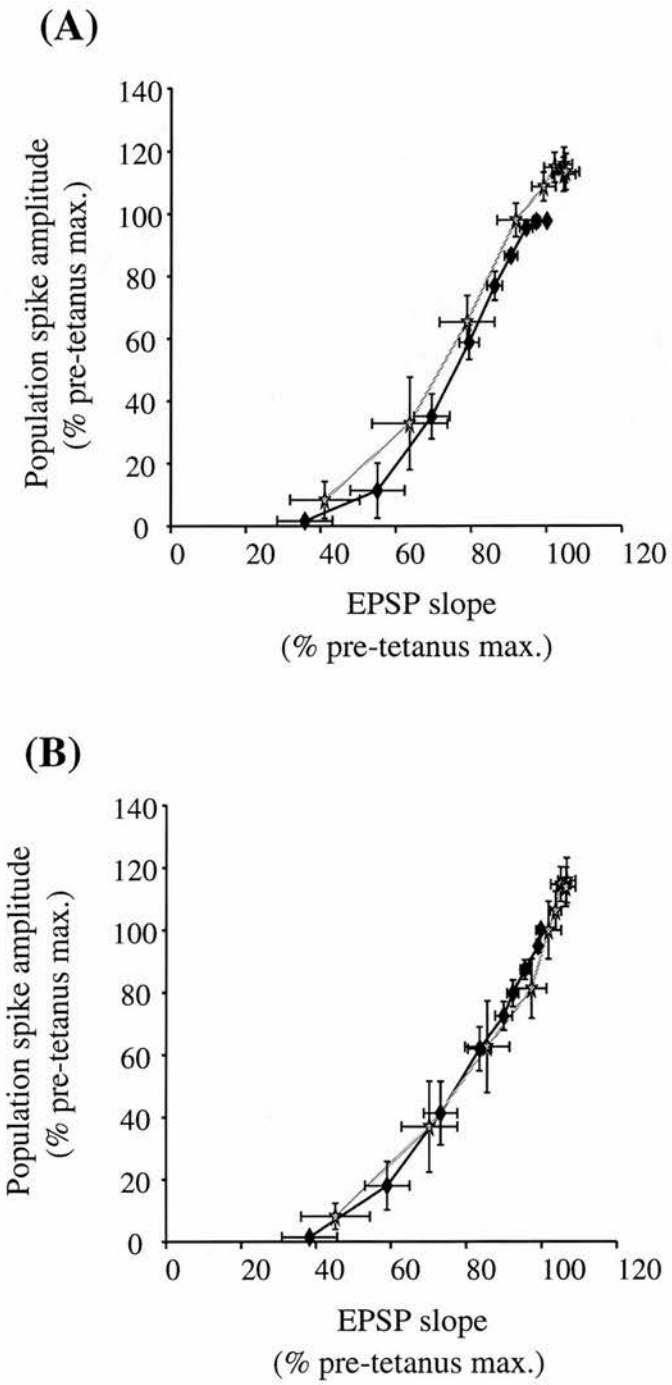


Fig. 8.4.2

Mean relationship between population spike amplitude and EPSP slope at each point on the I / O curve recorded before and after tetanization. Data from both groups, i.e. tetanus only (A) and tetanus + 1 Hz (B) are plotted (see text for details).

8.5 General discussion

The present experiment reveals that dentate LTP *in vivo* exhibits a limited vulnerable phase, lasting less than 10 min after induction, during which potentiation can be disrupted by 5 Hz stimulation. Neither the induction of LTP, nor its reversal, was blocked by the application of the mGluR antagonist, MCPG. Unlike the reversal of LTP, however, the reversal of tetanus-induced E-S potentiation occurred in a time-independent manner, and 5 Hz stimulation alone resulted in E-S depression.

8.5.1 The significance of theta frequency stimulation in LTP reversal

It may be significant that stimulation at 5 Hz, a frequency within the range of the hippocampal theta rhythm (see chapter 2.4.8), was effective in reversing LTP, whilst 1 Hz stimulation had no effect. The firing of neurons in area CA1 and the dentate gyrus of behaving rats is characterized either by high frequency bursts known as complex spikes, or by the firing of single action potentials (Ranck Jr., 1973). Both forms of activity are frequently phase-locked to the theta rhythm (Hill, 1978; Bland et al., 1980; Otto et al., 1991). It has been suggested that the firing of complex spikes may represent a mechanism for the induction of a naturally occurring form of synaptic potentiation, whilst the firing of single pulses phase-locked to theta may serve to reverse such potentiation (Larson et al., 1993). In fact, it has recently been reported that the exploration of a novel environment, a process that causes an increase in power at theta frequencies, can reverse tetanically-induced dentate LTP (Xu et al., 1998b).

However, stimulation at theta frequencies readily induces seizure-like afterdischarges (Ben-Ari et al., 1979; Burdette et al., 1996). In the present study, activity of this kind was always observed during 5 Hz stimulation, but never during 1 Hz stimulation. It is possible that theta frequency stimulation may be effective in reversing LTP, not because such stimulation mimics endogenous patterns of hippocampal activity, but because oscillatory circuits within the hippocampus are "tuned" to theta frequencies, and can readily be driven to pathological states of activity by stimulation within this frequency range. As such, it is possible that LTP reversal by 5 Hz stimulation, rather than being a candidate mechanism for the active forgetting of information, may have more in common with various neuropathological events. In this respect, it may be significant that the induction of seizures has been reported to reverse LTP and to disrupt memory consolidation (Hesse and Teyler, 1976; McGaugh, 1966).

Indeed, Abraham et al. (1996) reported that the delivery of 3 Hz stimulation starting 1 min after tetanization resulted in an apparent depotentiation of dentate LTP in awake rats, only when

accompanied by afterdischarge activity. No effect was obtained in one rat that did not exhibit afterdischarge activity. Furthermore, this "depotentiation" was found to be transient, and was interpreted as a non-specific depression of responses resulting from seizure generation. However, this possibility cannot explain the results of the present study, as afterdischarges were equally severe irrespective of the interval between tetanus and 5 Hz, yet no reversal of LTP could be induced 10 min or 30 min after tetanization.

Despite the fact that a general depression of EPSPs cannot explain the time-dependent reversal of LTP, it is impossible to rule out the possibility that afterdischarges play a necessary role in this phenomenon, but are not in themselves sufficient to reverse LTP. This issue can only be resolved if a means can be found of delivering a train of 5 Hz *in vivo* without inducing afterdischarges. For instance, it may be possible in future experiments to gradually increase stimulation frequency to 5 Hz without reaching the afterdischarge threshold. However, pilot studies using this technique have so far proved unsuccessful. Alternatively, the stimulation intensity might be gradually increased during 5 Hz stimulation, without eliciting seizure-like activity.

Of course, the simplest method of eliminating afterdischarges would be to use a very low stimulation intensity throughout 5 Hz stimulation. However, this method of stimulation would almost certainly activate fewer of the afferent fibres than those recruited during a high frequency tetanus of sufficient intensity to induce lasting LTP. Hence, it is likely that the use of very low stimulation currents during 5 Hz stimulation would fail to reverse LTP, and would provide no evidence about the role of afterdischarges in this phenomenon.

8.5.2 Changes in E-S coupling

In the present study, tetanization resulted in a modest E-S potentiation, a phenomenon that is routinely observed after tetanic induction of LTP, and has been attributed to a reduction in tonic GABAergic inhibition (Chavez-Noriega et al., 1990) and an enhanced activation of dendritic voltage sensitive calcium channels (Vida et al., 1995). The delivery of 5 Hz stimulation following tetanization reversed both LTP and E-S potentiation. The time-dependent reversal of LTP suggests that this phenomenon is probably input-specific. However, the reversal of E-S potentiation occurred in a time-independent fashion. Furthermore, the delivery of 5 Hz alone caused a decrease in E-S coupling. This suggests that 5 Hz stimulation causes a decrease in granule cell excitability in response to activity at both naïve and potentiated synapses, an action which casts doubt upon the input specificity of the time-independent reversal of E-S potentiation. The possible relevance of this finding to the use of 5 Hz stimulation as an alternative to drug treatment in behavioural studies is discussed below.

8.5.3 The mechanism of time-dependent LTP reversal

It is possible that the time-dependent reversal of LTP by 5 Hz stimulation may be dependent on the activation of adenosine A_1 receptors. 5 Hz stimulation is known to result in an increase in adenosine release from hippocampal slices (Jonzon and Fredholm, 1985; Cunha et al., 1996). It has been reported that the application of adenosine A_1 receptor antagonists to CA1 slices prevents the reversal of LTP by 5 Hz or 1 Hz stimulation in CA1 slices (Larson et al., 1993; Fujii et al., 1997). Furthermore, LTP can be reversed by the application of adenosine within 1 min, but not 5 min after tetanization (Arai et al., 1990b). This time window is similar to the period during which LTP was found to be vulnerable to reversal by 5 Hz stimulation in the present study. (However, Mitchell et al. (1993) have reported a failure to reverse CA1 LTP by the post-tetanus application of adenosine. The reason for this discrepancy is unknown).

It has recently been reported that LTP induced by theta burst stimulation in CA1 slices can be reversed in a time-dependent manner by the delivery of additional theta burst trains, a result which explains the inverted U-shaped relationship between the number of theta burst trains in a tetanus and the subsequent magnitude of LTP (Abraham and Huggett, 1997). As in the present study, the period during which LTP was vulnerable to reversal lasted less than 10 min. The application of an adenosine A_1 receptor antagonist prevented the reversal of LTP by excessive theta burst stimulation and allowed LTP to develop normally.

Interestingly, it may be significant that both adenosine A_1 receptor activation and low frequency stimulation are associated with a decrease in E-S coupling in area CA1 and the dentate gyrus, respectively (Stone and O'Kane, 1997; Bramham and Srebro, 1987). These results are reminiscent of the E-S depression reported here after the delivery of 5 Hz stimulation without prior tetanization.

The mechanism by which adenosine A_1 receptor activation can reverse LTP is unknown, although a number of possibilities have been proposed (see Arai et al., 1990b; Abraham and Huggett, 1997). It is unlikely that a presynaptic reduction in glutamate release resulting from A_1 receptor activation (see Dunwiddie, 1985) could account for a time-dependent reversal of LTP. However, hippocampal A_1 receptors are located both pre and postsynaptically (Deckert and Jorgensen, 1988). Furthermore, the application of adenosine has been found to inhibit cAMP accumulation in CA1 pyramidal cells (van Calker et al., 1979), a phenomenon subsequently found to be mediated by postsynaptic A_1 receptors. (Dunwiddie and Fredholm, 1989). Hence, is possible that A_1 receptor activation disrupts postsynaptic cAMP-dependent second messenger cascades known to be involved in the stabilization of LTP (Frey et al., 1993; Huang and Kandel, 1994).

However, there is also evidence that adenosine receptor activation inhibits the activation of integrins, a class of cell surface receptor (Thiel et al., 1996). In a recent study, an integrin antagonist was found to induce a time-dependent reversal of LTP in CA1 slices: application of the drug 25 min or more

after tetanization had no effect on potentiation (Stäubli et al., 1998). Integrins are involved in the control of morphological synaptic changes and the rearrangement of the postsynaptic membrane environment following LTP induction. Previous studies have implicated these receptors in the stabilization of LTP (Xiao et al., 1991; Bahr et al., 1997), and the inhibition of integrin activation provides a potential mechanism for the depotentiation induced by low frequency stimulation.

Hence, the results of the present study might be explained by the possibility that activation of A_1 receptors in response to adenosine release during 5 Hz stimulation disrupts second messenger cascades and / or integrin function during a vulnerable period of LTP stabilization, resulting in a time-dependent reversal of potentiation.

However, a number of questions remain unanswered in this scheme. In particular, the role of NMDA receptors in the time-dependent reversal of LTP is unknown. Furthermore, it is possible that the reversal of established LTP reported by some groups (e.g. Bashir and Collingridge, 1994; Doyle et al., 1997) is mechanistically distinct from the time-dependent reversal of LTP reported in the present study and elsewhere (e.g. Larson et al., 1993; Stäubli and Chun, 1996). One piece of evidence suggests that this might indeed be the case. It was mentioned above that adenosine can reverse LTP if applied within minutes of LTP induction, a phenomenon implicated in the time-dependent reversal of LTP by low frequency stimulation (Arai et al., 1990b). However, endogenous adenosine has been reported to attenuate both LTD and the depotentiation of established LTP in CA1 slices (de Mendonça et al., 1997). It is possible that the former result reflects a disruption of processes involved in the stabilization of LTP, whilst the latter result may reflect an inhibition of mechanisms involved in the induction of synaptic plasticity. Note that endogenous adenosine is found to inhibit LTP, in addition to its effects on LTD and depotentiation (Mendonça and Ribeiro, 1994).

It would be interesting to carry out a pharmacological characterization of the LTP reversal obtained in the present study. Unfortunately, the limited time window during which dentate LTP can be reversed would make such an undertaking very difficult. A ventricular infusion starting immediately after tetanization would be unlikely to result in a sufficient hippocampal drug concentration 2 min later. Direct intrahippocampal drug infusion might be possible, although it is likely that infusion at the rapid rate necessary would result in a general depression of EPSPs, thus confounding the results. Future studies will assess the practicality of such an approach.

8.5.4 LTP reversal by 5 Hz stimulation: physiological mechanism or experimental tool?

The physiological relevance of long trains of low frequency stimulation, such as a 10 min period of 5 Hz, is questionable. Nevertheless, the massive synchronous activation of afferent fibres occurring during high frequency tetanization is similarly unnatural. However, brief periods of theta burst

stimulation induce LTP and are physiologically plausible (see chapter 2.4.8). The possibility that LTP induced using a more physiologically realistic tetanus might be reversed by a brief period of theta stimulation remains a topic for future experiments. Nevertheless, regardless of the physiological significance of the present findings, the ability to preferentially erase recently induced LTP, whilst sparing established LTP, might provide a novel alternative to pharmacological intervention in future behavioural studies of synaptic plasticity and learning.

In one possible version of such a study, a rat would be implanted with chronic stimulation and recording electrodes, and trained in the watermaze delayed matching-to-place task (see Steele and Morris, 1999). At variable intervals after the first trial of a session, 5 Hz stimulation would be delivered. Retention of the platform position presented in the first trial would then be assessed. In this way, it could be determined whether stimulation known to reverse LTP in a time-dependent manner might also erase memory in a similar way.

However, there are several potential problems with the study proposed above. One such issue is the difficulty of activating a large septotemporal portion of the hippocampus by perforant path stimulation. It has been found that only a small residual fraction of the total hippocampal volume is sufficient for normal watermaze learning, as long as this portion is located in the dorsal hippocampus (Moser et al., 1993c, 1995). Hence, any treatment affecting a limited region of the hippocampus only is unlikely to succeed. This problem has dogged research into the behavioural effects of LTP saturation, but has recently been overcome by the implantation of recording electrodes into the dorsal hippocampus following lesioning of the contralateral hippocampus (Moser et al., 1998). Tetanization was delivered via an array of stimulating electrodes straddling the angular bundle of the perforant path. Although laborious, such a technique would probably be necessary in a behavioural study of memory erasure by 5 Hz stimulation.

A further issue concerns the synapse specificity of the LTP reversal effect. This was not conclusively proven in the present study, although the time-dependent nature of LTP reversal provides some support for this interpretation. However, the assumption that the effects of 5 Hz stimulation are limited to the reversal of recently established LTP may not be strictly true. Ideally, this issue should be resolved by the use of dual input studies prior to any behavioural exploitation of the LTP reversal effect

The delivery of 5 Hz stimulation following tetanization reversed both LTP and E-S potentiation in the present study. However, the reversal of E-S potentiation, unlike the reversal of LTP, occurred in a time-independent fashion. Furthermore, the delivery of 5 Hz stimulation alone caused a decrease in E-S coupling. This suggests that 5 Hz stimulation can cause a decrease in granule cell excitability in response to activity at naïve synapses, an action which casts doubt upon the input specificity of the time-independent reversal of E-S potentiation following a tetanus. It is possible that a non input-specific reduction in granule cell excitability might impair distant as well as recent memories.

However, there is no reason to suppose that the degree of E-S depression would vary with the interval between learning and 5 Hz stimulation. Indeed, the reversal of E-S potentiation induced by a tetanus occurred in a time-independent manner as mentioned above. Hence the possibility that 5 Hz stimulation might cause a decrease in E-S coupling in a behaving rat would not be likely to obscure a delay-dependent effect on recall, even if an overall degradation of memory were found.

Finally, 5 Hz stimulation was always associated with epileptiform afterdischarges, phenomena which may well have adverse physiological consequences in a behaving rat. It would be desirable to avoid such pathological activity in any study of the effects of 5 Hz stimulation on learning.

In conclusion, the objections raised above do not rule out the possibility of using low frequency stimulation as a way of preferentially erasing recent synaptic potentiation, although such a study would be technically very demanding. However, the principle of looking for a tool which allows the selective targeting of potentiated synapses may well be characteristic of future research (see Barnes, 1995).

Chapter Nine

Factors associated with the magnitude of LTP

9.1 Introduction

Throughout the period during which the work described in previous chapters was carried out, intermittent problems with LTP induction were experienced. Typically, a tetanus that had previously induced robust and lasting LTP would, a few months later, under apparently identical conditions, induce only a short-term potentiation which would return to baseline within an hour. The problem would spontaneously resolve itself again after another one or two months, with robust LTP being obtained using the same recording and tetanization parameters. It was noticed that periods during which LTP induction failed tended to occur in the winter, rather than the summer, an issue which is discussed later. On a number of occasions, experiments were postponed after pilot studies revealed a failure to induce lasting LTP of sufficient magnitude. In fact, the use of different tetanus parameters in chapters 6, 7 and 8 was determined by the need to overcome difficulties in LTP induction.

Owing to the practical importance of this issue, efforts were made to identify some of the factors governing the magnitude of LTP. Whilst this analysis did not entirely solve, or indeed explain, the problems in LTP induction, it served to highlight the critical importance of a number of baseline stimulation and tetanization parameters which are generally set at arbitrary values. The organization of this chapter is partly chronological and partly thematic. This structure is necessary in order to explain the rationale behind the data analysis and pilot studies carried out at each stage.

9.2 The influence of initial EPSP magnitude on subsequent LTP: further analysis of data from experiment 6.2

9.2.1 Introduction

In experiment 6.2, an attempt was made to determine the effects of MCPG on LTP induction. However, this attempt was unsuccessful owing to the failure to induce lasting LTP in either drug-treated or control groups. The tetanus used in this study (see chapter 6.2.1) had been successfully used in studies of LTP in freely moving rats (Riedel et al. 1994a, 1995a). However, stronger tetanization parameters may be needed to induce LTP under urethane anaesthesia (see chapter 3.10.5 & 7.7).

Nevertheless, during the study it appeared that the "better" the original EPSP after implantation of electrodes, the less LTP was obtained after tetanization. In order to quantify this subjective impression, the correlation between LTP magnitude and a range of baseline parameters was investigated. Data from drug and control groups (total n = 24) were pooled for the following analysis, because no significant differences between groups were observed.

There are a number of possible measures of "initial" EPSP slope magnitude that might be used, e.g. the baseline EPSP slope recorded over the 10 min prior to tetanization, or the EPSP slope recorded at a fixed stimulation intensity during the initial I / O curve before the start of the main experiment (see chapter 6.2.1.5). In fact, the maximum EPSP slope recorded during this I / O curve was initially chosen, although the pre-tetanus baseline EPSP slope was also analysed.

9.2.2 Results

Despite the adjustment of test pulse stimulation intensity, the pre-tetanus baseline EPSP slope was very significantly correlated with the maximum EPSP slope elicited during the initial I / O curve [figure 9.2.1; $r = 0.83$; $p < 0.01$], suggesting that both measures are broadly equivalent. However, this correlation indicates that the attempt to standardize EPSPs by adjusting the baseline stimulation intensity was only partially successful.

Slope LTP was significantly negatively correlated with maximum initial slope when measured both 50-60 min post-tetanus [figure 9.2.2A; $r = -0.55$; $p < 0.01$] and 110-120 min post tetanus [figure 9.2.2B; $r = -0.63$; $p < 0.01$], confirming the initial suspicions that "good" potentials result in poor LTP. No significant correlation was found between the level of EPSP slope PTP (0-4 min post tetanus) and maximum initial EPSP slope [$r = -0.18$; NS; data not shown]. The baseline EPSP slope

recorded over the 10 min before tetanization was also found to be significantly correlated with the amount of EPSP slope LTP 50-60 min and 110-120 min after tetanization [$r = -0.47$; $p < 0.05$ and $r = 0.44$; $p < 0.05$ respectively; data not shown].

In addition to the above correlations, EPSP slope LTP measured 110-120 min after tetanization was found to be negatively correlated with maximum initial population spike amplitude [figure 9.2.3A; $r = -0.58$; $p < 0.01$] and maximum initial EPSP amplitude [figure 9.2.3B; $r = -0.51$; $p < 0.02$].

No significant correlations were found between the initial maximum values of each measure (EPSP slope, population spike, or EPSP amplitude) and population spike PTP and LTP at any time point (data not shown). No significant correlations were found between baseline stimulation intensity and the PTP or LTP of any measure (data not shown). Similarly, baseline stimulation intensity was correlated neither with the maximum EPSP slope obtained during the initial I / O curve, nor the pre-tetanus baseline EPSP slope (data not shown).

In order to investigate the possible relationship between learning ability and the subsequent induction of LTP, data from (-)-MCPG and 20 mM MCPG-treated rats were pooled (total n = 14), owing to the absence of any difference in watermaze performance between these two groups. However, percentage time in the training quadrant during the transfer test was not significantly correlated with the level of EPSP slope LTP recorded either 1 hr or 2 hr after tetanization [$r = 0.03$ and 0.08 respectively; data not shown].

9.2.3 Discussion

The suspicion that "good" EPSPs result in poor LTP was confirmed by an analysis of the negative correlation between various initial attributes of an EPSP, and the level of LTP subsequently induced.

It is possible that the initial size of an EPSP reflects naturally occurring synaptic enhancement as a result of watermaze learning. If so, the failure to potentiate initially large EPSPs may be a consequence of the fact that such EPSPs are already substantially potentiated above baseline levels. However, this explanation predicts a negative correlation between watermaze learning and LTP. In fact, no significant correlation was found between the percentage time spent in the training quadrant during the transfer test, and the level of LTP 1 or 2 hr after tetanization.

However, it is likely that differences in the placement of stimulation and recording electrodes play a role in determining the level of LTP induced. The accuracy with which the stimulating electrode is lowered into the angular bundle of the perforant path will determine how many fibres are activated by test pulse stimulation. An accurate electrode placement that activates a large number of fibres will generate an EPSP with a large population spike and EPSP slope. This effect is not cancelled out by

the adjustment of test pulse stimulation intensity in order to elicit a population spike 40 % of the maximum obtainable. Pre-tetanus baseline EPSP slope values were still found to correlate significantly with initial maximum values (figure 9.2.1).

Jeffery (1995) reported that if I / O curves were measured in freely moving rats, before and after tetanization, the greatest amount of LTP was seen at the lowest test pulse intensities. It was suggested that non-linear summation of post-synaptic potentials might explain this phenomenon. As more perforant path fibres are recruited, the number of converging inputs to dentate granule cells increases, which leads to a rise in non-linear summation. Thus, at high test pulse intensities, non-linear summation may result in a smaller percentage EPSP slope increase than that observed using low test intensities, at which non-linear summation is negligible. A similar phenomenon may explain why large EPSPs, presumably entailing the activation of a high number of perforant path afferents, show less LTP after tetanization than smaller EPSPs, in which considerably fewer converging inputs are activated. However, it is unlikely that non-linear summation of EPSCs can account for a total failure of LTP in a large proportion of cases. Ideally, plots of LTP across a range of test pulse intensities should be constructed, in order to directly compare the present findings with those of Jeffery (1995). However, this analysis is not possible using the present data, owing to I / O curves having been recorded only at the start of the experiment. A more thorough analysis is presented below based on data obtained in experiment 7.3.

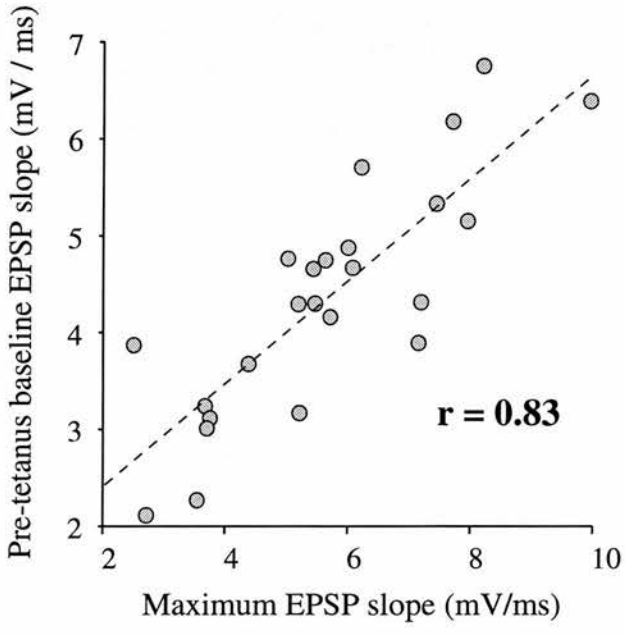


Fig 9.2.1

(A) Pre-tetanus baseline EPSP slope is highly correlated with the maximum EPSP slope recorded during the initial I / O curve.

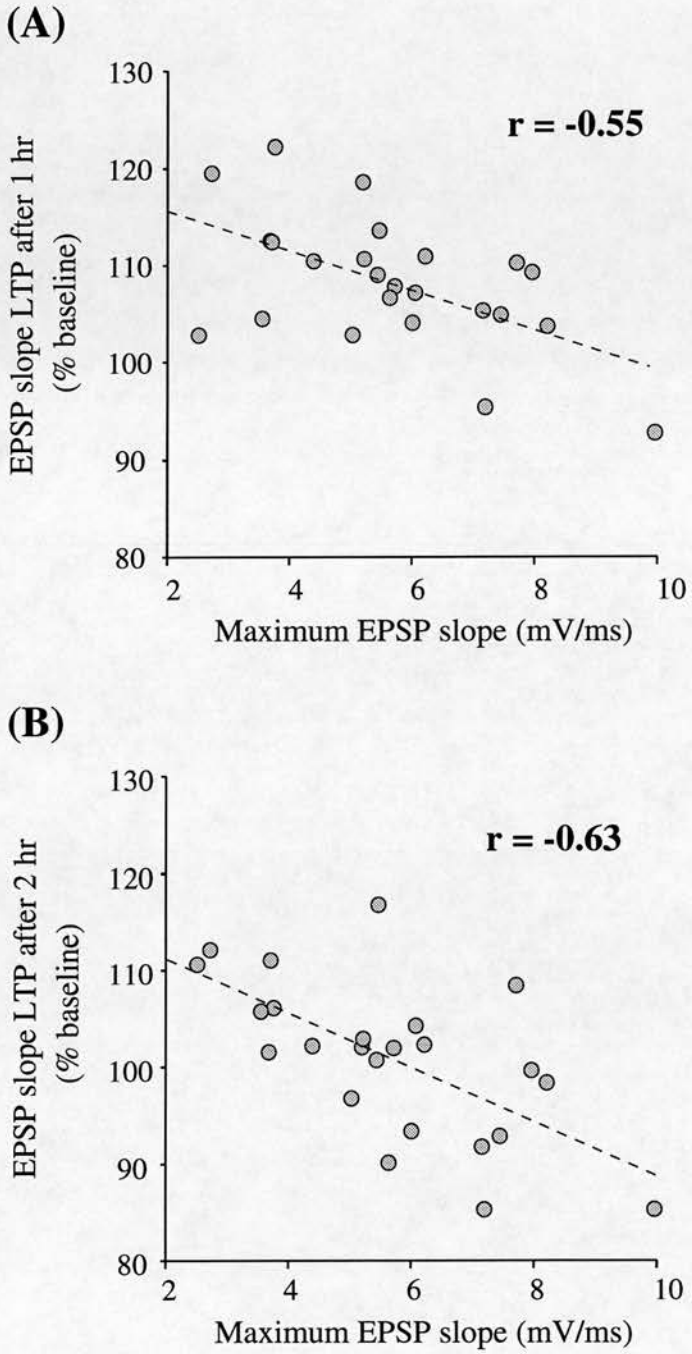


Fig. 9.2.2

(A) EPSP slope LTP 1 hr after tetanization is negatively correlated with maximum EPSP slope during an initial I / O curve.
(B) EPSP slope LTP 2 hr after tetanization is also negatively correlated with maximum EPSP slope.

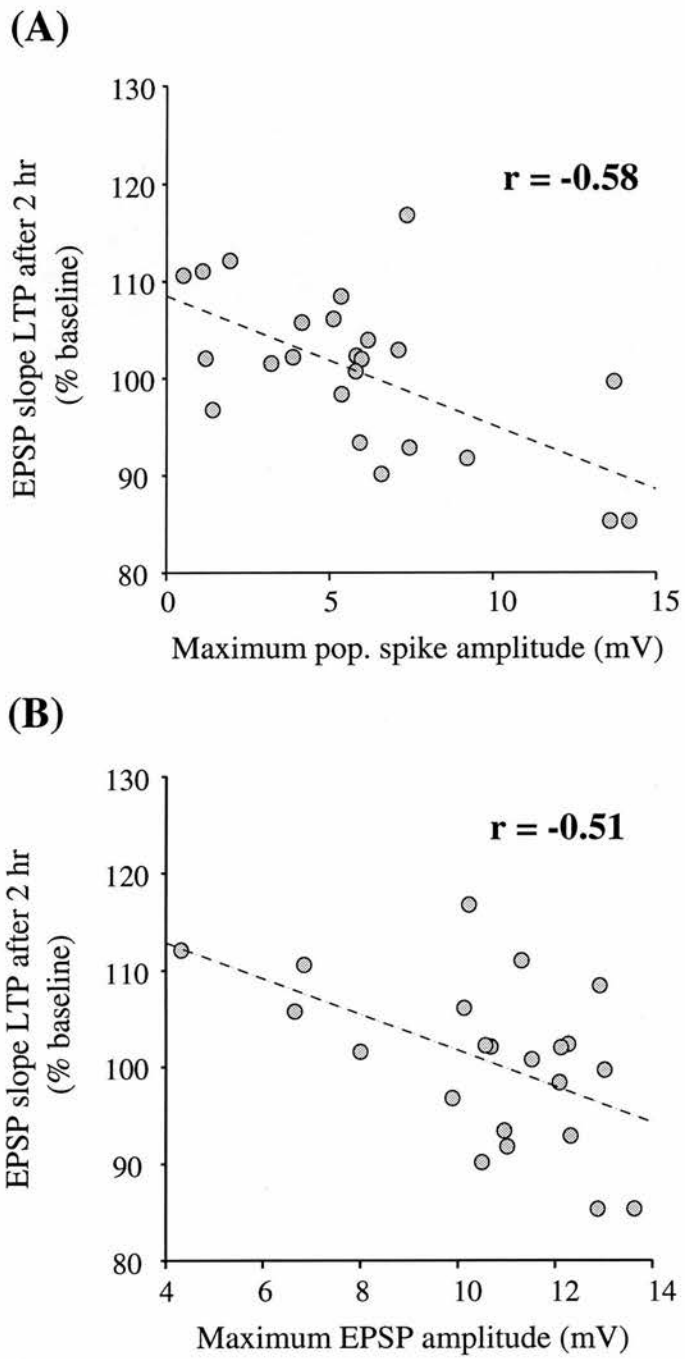


Fig. 9.2.3

(A) EPSP slope LTP 2 hr after tetanization is negatively correlated with maximum initial pop. spike amplitude
(B) EPSP slope LTP 2 hr after tetanization is also negatively correlated with maximum initial EPSP amplitude.

9.3 The effect of tetanus intensity and initial EPSP magnitude on EPSP slope LTP: pilot studies and further analysis of data presented in experiment 7.3

9.3.1 Pilot studies

The failure to induce LTP at the end of watermaze testing in experiment 6.2 was solved by the use of a different tetanus that induced lasting LTP in control rats (see experiments 7.3, 7.4 and 7.6). However, during the autumn and winter following the completion of the work described in chapter 7, this tetanus induced little or no LTP. Although the reasons for the change in effectiveness are unknown, it is possible that a larger current during tetanization might result in greater afferent cooperativity and hence greater LTP. Indeed, McNaughton et al. (1978) reported that a threshold exists for the induction of LTP, and that even above this threshold, the amount of LTP increases as the tetanization intensity is increased.

In order to examine whether a more intense high frequency tetanus might result in LTP, a number of rats were tetanized either at double the baseline test pulse intensity (n = 6), or with a 1 mA tetanus regardless of the test pulse intensity (n = 7). Potentiation was recorded for 1 hr after tetanization. The tetanus and recording protocol used was otherwise identical to that described in experiment 7.3. In both conditions, little or no lasting EPSP slope LTP was induced (figure 9.3.1A). No group difference in EPSP slope potentiation 1 hr after tetanization was found [$F < 1$]. Test pulse stimulation intensities were equivalent between groups [$F(1,11) = 1.09$; $p > 0.3$; data not shown].

However, population spike amplitudes were still elevated 1 hr after tetanization (figure 9.3.1B). The increase was modest (approximately 2 mV), and did not differ between groups [$F < 1$]. It is interesting that population spike potentiation was more robust than EPSP slope potentiation, a phenomenon which was often noted in the course of LTP experiments. In fact, since almost no slope LTP remained after 1 hr in the present study, the only persistent change in these animals was an E-S potentiation. A similar transient potentiation of the EPSP slope, but a lasting potentiation of the population spike has been reported by others under some circumstances (e.g. Pugliese et al., 1994).

Owing to the fact that I / O curves were recorded before and after tetanization in this study, it was possible to examine the amount of LTP induced at a range of test pulse intensities, independently of the test pulse stimulation intensity used throughout the main experiment. As mentioned above, this analysis was not possible in experiment 6.2, owing to the fact that I / O curves were recorded before tetanization only. Figure 9.3.2A shows the amount of LTP induced over the entire range of test pulse intensities sampled in the I / O curves recorded 20 min before and 1 hr after tetanization. A detailed account of this form of analysis is given in experiment 7.3. Little LTP was induced at any test intensity in either group, but potentiation was lowest at the smallest test intensity, 0.1 mA (see section

9.3.2).

The above data reveal that at times during which problems are experienced with LTP induction, simply increasing the tetanus intensity does not provide a solution.

9.3.2 Further analysis of data from experiment 7.3: Effect of tetanus intensity on LTP across the I / O curve

In order to investigate the phenomenon of EPSP slope depression at low test intensities in more detail, it was decided to re-examine data from experiment 7.3. Note that in this experiment, I / O curves were recorded 20 min before and 2 hr after tetanization. Owing to the absence of a difference between drug and vehicle groups, data from all 20 rats in experiment 7.3 were pooled for the following analysis. Figure 9.3.2B shows the mean EPSP slope potentiation over the entire range of test intensities. Note the slight depression and increased standard error at a test intensity of 0.1 mA, a phenomenon that is also observed in figure 9.3.2A.

Figure 9.3.3 shows sample plots relating LTP to test pulse intensity in three individual rats. In rat 1, the greatest amount of potentiation was obtained at low test intensities. However the data from rats 2 and 3 are perhaps more typical. In these animals, the largest potentiation was seen at high test intensities. In fact, tetanization caused a lasting depression of EPSP slope in rat 3 at the two lowest test intensities. The raw I / O curve data from rats 1 and 3 are shown in figure 9.3.4. The percentage LTP at each test intensity displayed in figure 9.3.3 was obtained simply by dividing the EPSP slope value after tetanization by the value recorded before tetanization, then multiplying by 100. Note that the pre- and post-tetanus I / O curves for rat 3 cross over at a test intensity of about 0.3 mA. Prior to this point, EPSP slope values are depressed following tetanization, a phenomenon that is small in absolute terms, but large in percentage terms.

Since baseline test pulse intensities in experiment 7.3 were set at different values in each rat in order to elicit a population spike amplitude of 2-4 mV, and since the tetanization current was always set at double this value, a wide range of tetanus intensities was used. Hence, using I / O curve data, the effect of tetanus intensity on the magnitude of LTP can be investigated over a range of test intensities.

In order to assess the overall relationship between tetanus intensity, test pulse intensity, and LTP magnitude, scatter plots were constructed relating EPSP slope LTP to tetanus intensity at each test pulse intensity sampled during the recording of I / O curves. Scatter plots of this relationship at 0.1 mA, 0.5 mA, and 1.0 mA are shown in figure 9.3.5. Significant correlations were revealed between tetanus intensity and LTP magnitude only at the lowest test intensities. The change in the value of the correlation coefficient, *r*, of the relationship between EPSP slope LTP and tetanus intensity as test pulse intensity was increased is shown in figure 9.3.6A. The slope of this function became

progressively less negative as test pulse intensity was increased. Significant values of r are indicated (see figure legend). At face value, this result suggests that tetanus intensity is negatively correlated with LTP magnitude only at low test pulse intensities. However, the interpretation of these data is complicated by the fact that tetanus intensity was found to be significantly negatively correlated with initial maximum EPSP slope [r = -0.52; $p < 0.05$; figure 9.3.6B], i.e. small EPSPs were tetanized more strongly than large EPSPs. This correlation is an artifact of the adjustment of test pulse stimulation intensity to elicit a population spike of fixed amplitude. If the EPSP slope and population spike amplitude were initially small, the baseline test pulse intensity was set at a high value in order to elicit an EPSP of standard size. Since the tetanus intensity was always set at double the test pulse intensity, small EPSPs were tetanized more intensely than large EPSPs. Note that no such relationship was found in data taken from experiment 6.2, a finding which may be explained by the use of different criteria for the setting of baseline test pulse intensity.

Because initial maximum EPSP slope is a factor likely to influence the level of LTP obtained (see section 9.2), partial correlations were calculated. At each test pulse intensity, the relationship between tetanus intensity and LTP was assessed, whilst controlling for the effects of initial maximum EPSP slope. The partial correlation coefficient at each test pulse stimulation intensity is shown in figure 9.3.6C. Note that the correlation between tetanus intensity and LTP is now negative at all test pulse intensities. A non-significant trend towards a negative correlation was obtained at several intensities, and the correlation reached significance at 0.7 mA (see figure legend).

The present results reveal that, over the range tested, tetanization strength is inversely correlated with the amount of EPSP slope LTP. Note that although the strength of the corrected negative correlation was equivalent at all test intensities, an EPSP slope depression was only observed at the lowest test intensities, and good LTP was usually obtained at high intensities (see figure 9.3.2B). The reason for the depression of EPSPs at the low end of the I / O curve is not known. One possibility, however, is that feed-forward inhibition may be potentiated after tetanization. It has been reported in area CA1 that at low stimulation levels, the magnitude of the IPSP dominates EPSP components, but that a larger number of excitatory components than inhibitory components are recruited with increasing test stimulation intensity (Turner, 1990). Hence, a potentiation of feed-forward inhibition would preferentially cause a depression at low test intensities. Furthermore, if tetanization strength determines the extent to which feed-forward inhibition is potentiated, the inverse correlation between tetanus strength and percentage LTP might be explained.

Results consistent with those of the present analysis have been described in the dentate gyrus of freely moving rats. Hodgson et al. (1997) found that a high intensity tetanus resulted in an EPSP slope depression at low test intensities, but EPSP slope potentiation at high test intensities. Low intensity tetanization, in contrast, resulted in LTP at low test pulse intensities. A potentiation of feed-forward inhibition by high intensity tetanization was suggested as an explanation for this pattern of

results. It was argued that fibre damage was not the cause of the EPSP depression resulting from high intensity tetanization, since depressed responses were found to recover over time. Furthermore, 4 weeks after the depression of EPSPs by high intensity tetanization, the application of a low intensity tetanus was able to induce LTP at the low end of the I / O curve.

The results of the present study and those of Hodgson et al. (1997) are somewhat different from those reported by Jeffery (1995) in freely moving rats. In the latter study, the effects of tetanus intensity were not investigated. However, the greatest amount of LTP was always observed at low test pulse intensities. Plots relating EPSP slope LTP to test pulse intensity generally resembled the data from rat 1 shown in figures 9.3.3 and 9.3.4 of the present study. It was found that after plotting percentage EPSP slope LTP against the log of test pulse intensity for each rat, the data were well described by a linear fit with negative slope. However, in the present study, such semi-log plots were found to be positive in 13 out of 20 rats tested (data not shown), reflecting the fact that LTP was usually smallest at low intensities. One possible reason for the discrepancies between studies is the fact that the tetanus intensities used by Jeffery (1995) were typically about 0.3 mA, i.e. about half those used in the present study. However, Jeffery (1995) used unanaesthetized rats, a factor which complicates such comparisons. Nevertheless, the high intensity tetanus used by Hodgson et al. (1997) was set at a current of 1.259 mA, higher than any tetanus used here.

Whatever the explanation for this phenomenon, in future experiments, baseline test pulse intensities should be set at levels not lower than 0.2 mA, and that the use of high intensity tetani should be avoided.

9.3.3 Further analysis of data from experiment 7.3: Effect of initial maximum EPSP slope on LTP across the I / O curve

In section 9.2, it was reported that, paradoxically, large EPSPs show a smaller percentage LTP than small EPSPs. Analysis of the relationship between maximum EPSP slope recorded during the initial I / O curve to percentage LTP at increasing test pulse intensities was carried out in exactly the same way as described for tetanus intensity in section 9.3.2. Maximum initial EPSP slope values recorded during the first I / O curve were highly correlated with pre-tetanus baseline EPSP slope values [r = 0.89; $p < 0.001$; data not shown], and with the EPSP slope recorded at all test pulse intensities [$p <$ 0.01 or less in all cases; data not shown]. This result illustrates the fact that, as in section 9.3.2, the maximum initial EPSP slope is broadly equivalent to all other alternative EPSP slope measures which might have been chosen as independent variables in the following analysis.

Maximum initial slope values did not correlate significantly with EPSP slope LTP at any test pulse intensity, although a weak, non-significant, positive association with EPSP slope LTP was observed

at low test intensities, a relationship which became negative at higher test intensities (but see below for analysis of partial correlations). Figure 9.3.7 shows scatter plots of the relationship between initial EPSP slope and LTP at test intensities of 0.1 mA (A), 0.5 mA (B), and 1.0 mA (C). The change in the correlation coefficient of the relationship between initial EPSP slope to percentage LTP is plotted in figure 9.3.8A. However, as mentioned above, maximum initial EPSP slope was significantly negatively correlated with tetanus intensity. Hence partial correlation coefficients were calculated relating initial maximum EPSP slope to LTP at each test pulse intensity, whilst controlling for the effects of tetanus intensity. The results are shown in figure 9.3.8B. Note that the corrected correlation between initial EPSP slope and LTP is always negative, but becomes progressively stronger as the test intensity is increased, reaching significance at 1.0 mA (see figure legend). The original weak positive relationship between initial maximum EPSP slope and percentage LTP at low test intensities was merely an artifact of the negative correlation between tetanus intensity and initial maximum EPSP slope values.

However, because the maximum initial EPSP slope was recorded at or near a test intensity of 1 mA, the fact that this measure is most strongly correlated with LTP at a 1 mA test intensity could be an artifact. In order to rule out this possibility, partial correlations between the initial EPSP slope elicited by stimulation at 0.1mA and percentage LTP at all test intensities were calculated, whilst controlling for the effects of tetanus intensity. Consistent with the results obtained using initial maximum EPSP slope, the strength of this correlation was greatest at high test intensities. Hence, the deleterious effects on LTP of a large initial EPSP slope are genuinely most severe at high test intensities.

The finding that initial EPSP slope is negatively correlated with percentage LTP is consistent with the results of section 9.2. It was suggested that the generation of a large EPSP is likely to involve the activation of a larger number of perforant path fibres than the generation of a small EPSP. Owing to the greater degree of postsynaptic convergence resulting from the activation of many afferent fibres, large EPSPs may suffer from a greater masking of LTP by non-linear summation of postsynaptic potentials than small EPSPs. According to this account, increasing the test pulse intensity would make matters even worse, by recruiting even more afferent fibres. This may explain the present observation that the negative correlation between initial EPSP slope and percentage LTP is strongest at high test pulse stimulation intensities.

However, variability in the initial size of an EPSP is inevitable, and EPSP magnitude is not a factor that can easily be manipulated. It was found in both the present section and section 9.2.2 that baseline EPSP slope is still highly correlated with initial maximum EPSP slope, even after an attempt has been made to standardize potentials by adjusting the test pulse stimulation intensity. Hence, even if a relationship exists between initial EPSP magnitude and LTP, there is little that could be done except to ensure that the mean initial EPSP slope is equivalent across experimental groups.

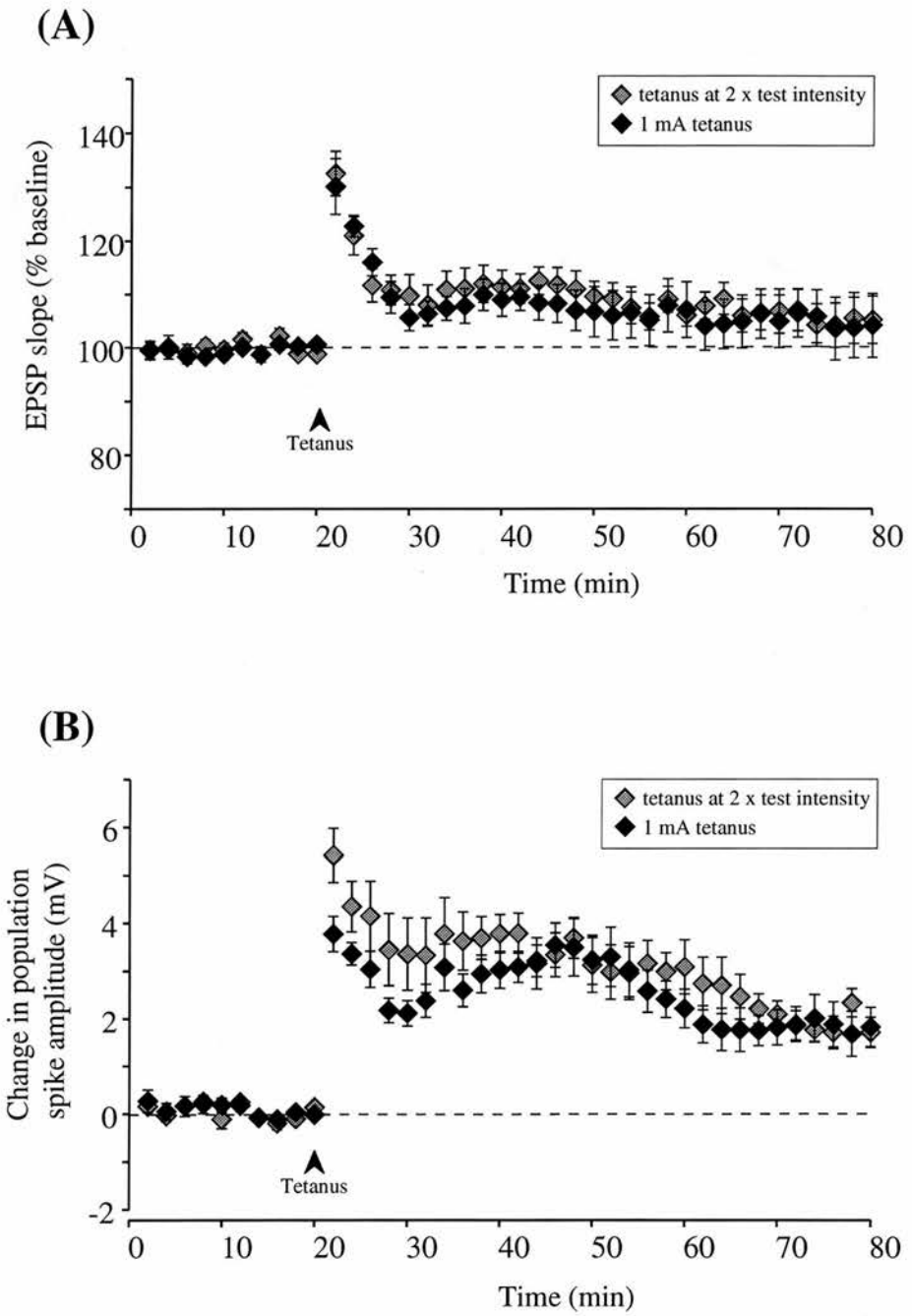


Fig. 9.3.1

Tetanization with 5 trains of 20 pulses at 250 Hz, 10 s between trains. Stimulation intensity was set at either double the baseline intensity (n = 6) or 1 mA (n = 7). No differences in EPSP slope LTP (A) or population spike LTP (B) were observed.

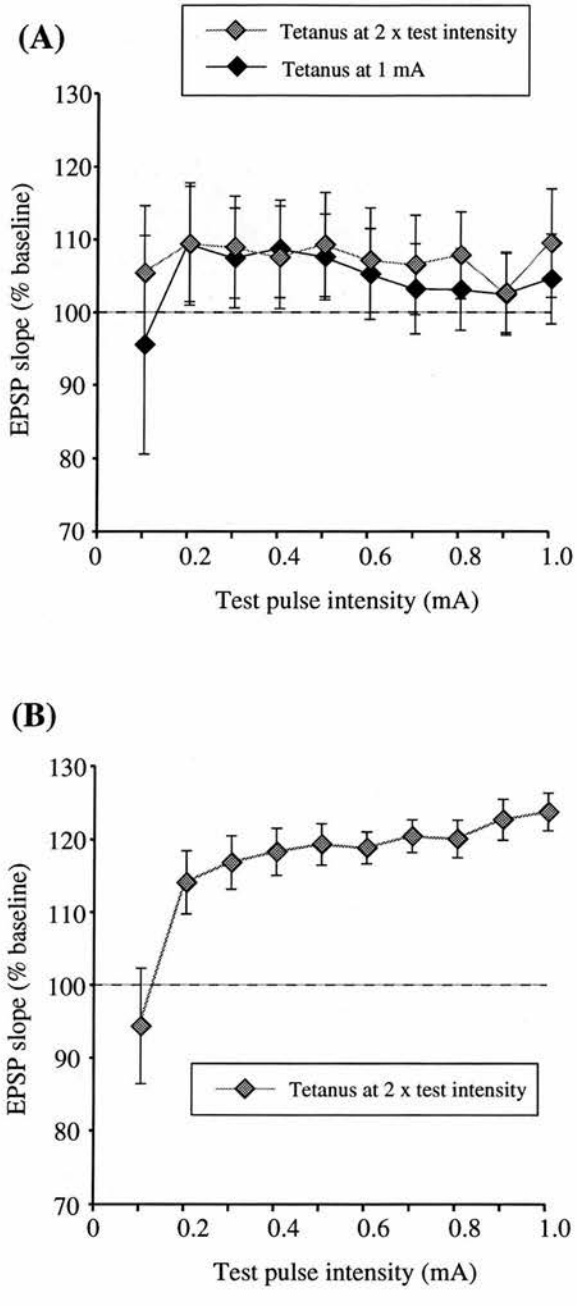


Fig. 9.3.2

Percentage EPSP slope LTP over the full range of test pulse intensities. (A) LTP pilot study in which little or no LTP was induced. (B) successful induction of LTP in experiment 7.1.

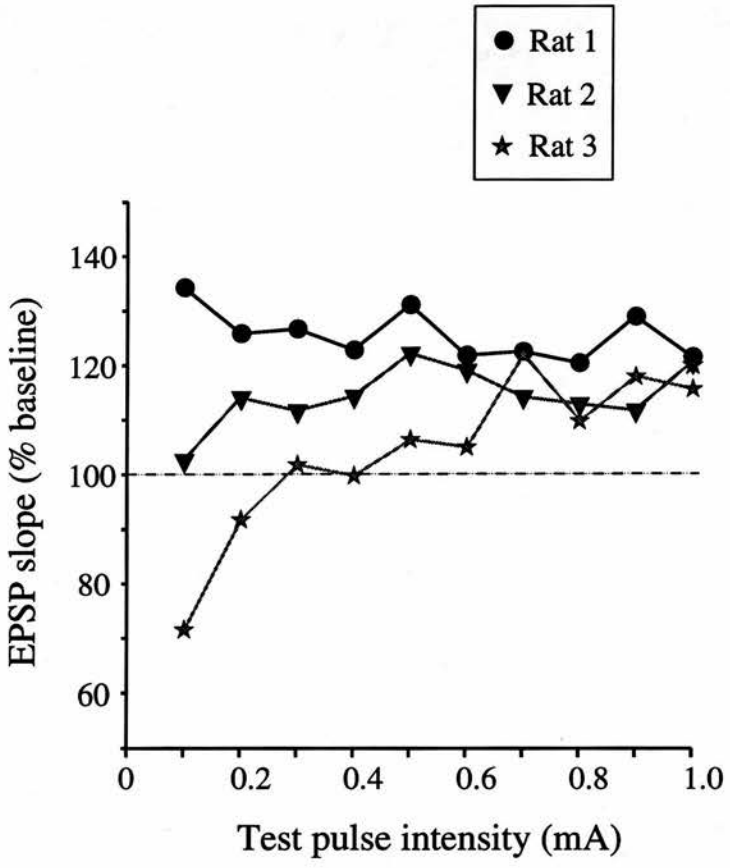


Fig. 9.3.3

Plots of EPSP slope LTP vs. test intensity in 3 individual rats.

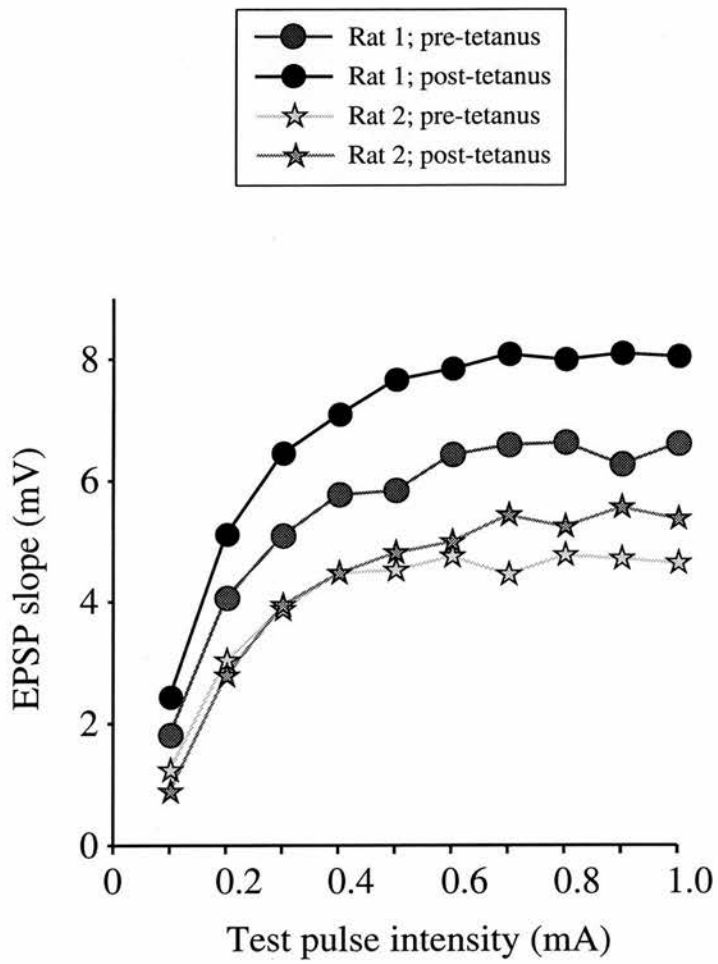


Fig. 9.3.4

The absolute increase in EPSP slope across areange of test pulse intensities. Rat 2 shows a slight depression in EPSP slope at low test intensities, but a potentiation at high test intensities.

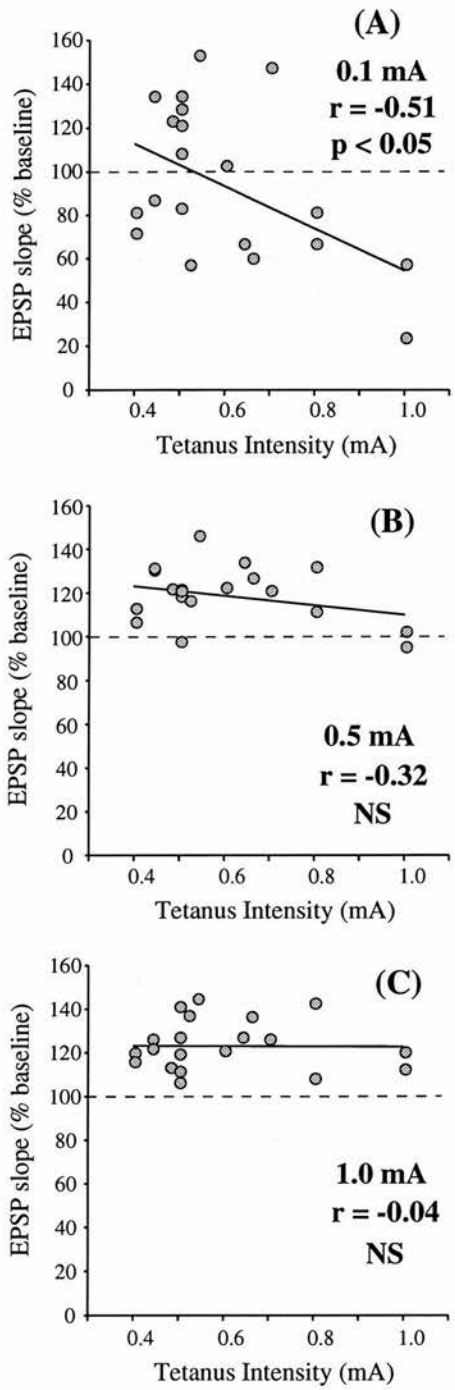


Fig. 9.3.5

The relationship between EPSP slope LTP and tetanus intensity at three different test pulse stimulation intensities. Data were calculated from I / O curves recorded 20 min before, and 2 hr after tetanization.

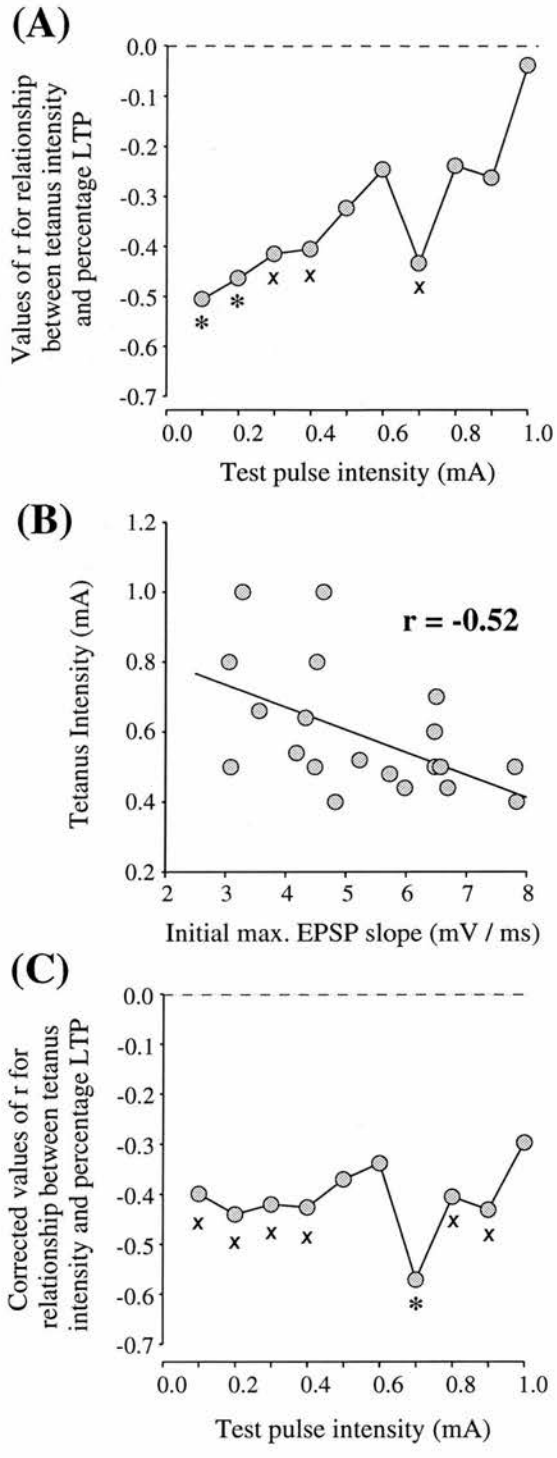


Fig. 9.3.6

(A) Values of correlation coefficient, r, obtained from regression lines relating percentage EPSP slope LTP to tetanus intensity over the full range of test pulse stimulation intensities. (B) Tetanus intensity is negatively correlated with initial maximum EPSP slope. (C) Values of partial correlation coefficient, r, obtained by relating EPSP slope LTP to tetanus intensity whilst controlling for the effects of initial maximum EPSP slope. [$^{*}p < 0.05$; $^{x}p < 0.1$]

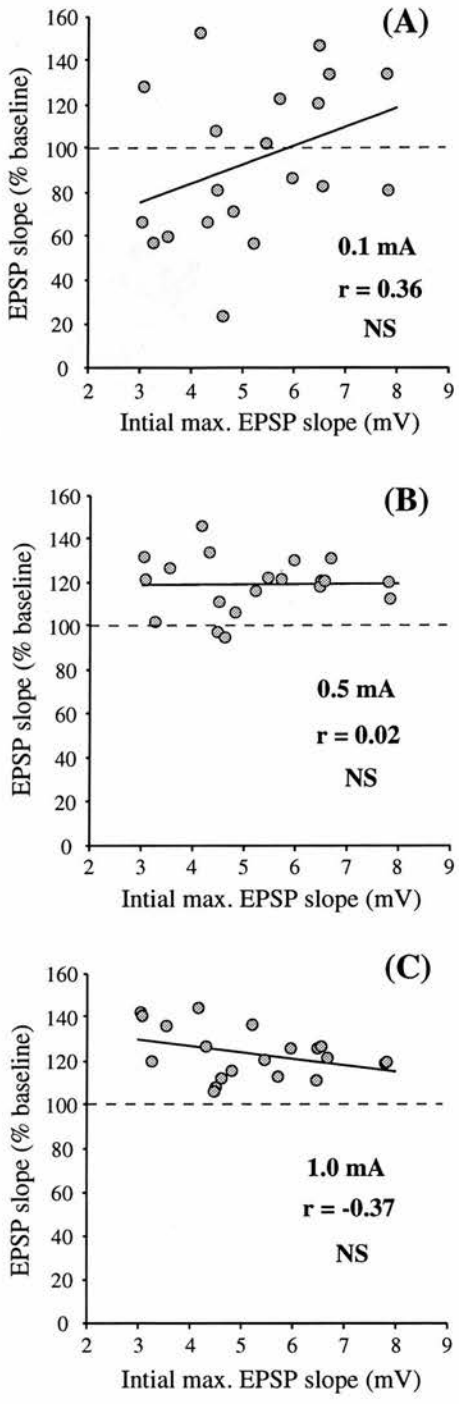


Fig. 9.3.7

The relationship between EPSP slope LTP and initial maximum EPSP slope at three different test pulse stimulation intensities. Data were calculated from I / O curves recorded 20 min before, and 2 hr after tetanization.

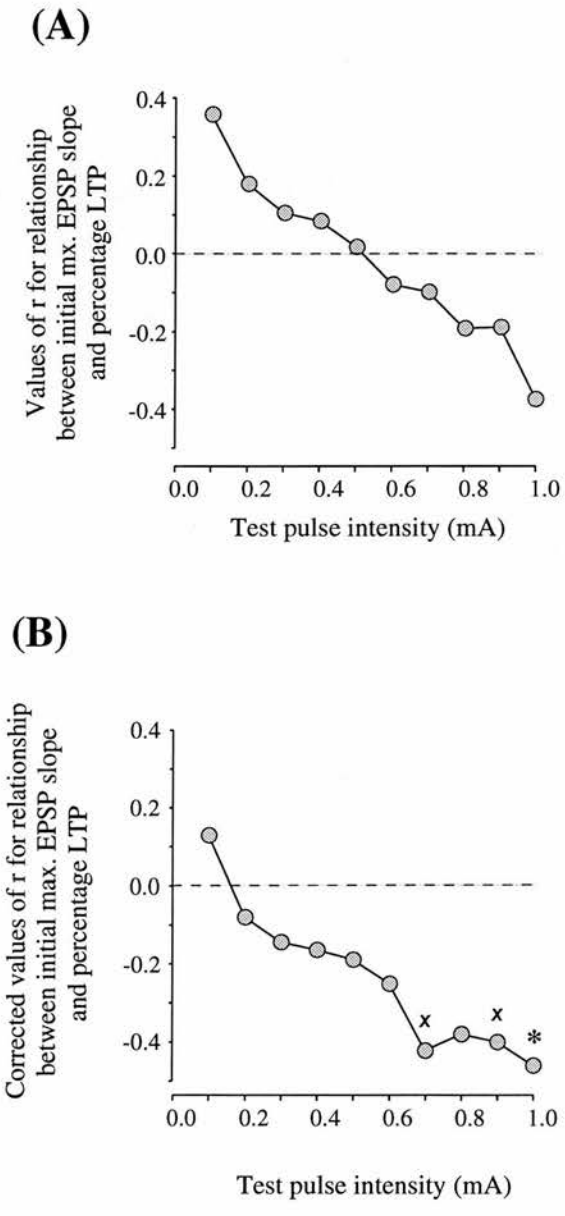


Fig. 9.3.8

(A) Values of correlation coefficient, r, obtained from regression lines relating percentage EPSP slope LTP to initial maximum EPSP slope over the full range of test pulse stimulation intensities.
(B) Values of partial correlation coefficient, r, obtained by relating EPSP slope LTP to initial maximum EPSP slope whilst controlling for the effects of tetanus intensity. [$^{*}p < 0.05$; $^{x}p < 0.1$]

9.4 Other variables associated with LTP magnitude

9.4.1 Introduction

This section begins with a description of a new set of baseline stimulation and tetanization parameters that were found to induce robust LTP, despite the failure of previously adequate parameters (see section 9.3.1). Possible reasons for the success of this protocol are discussed, after which a number of additional issues relating to the induction of LTP, such as the strain of rat used, stress levels, and seasonal variations in hormone levels are addressed.

9.4.2 The use of novel tetanus parameters: inter-train interval is only moderately important

The inability to induce robust LTP under conditions apparently identical to those of previous experiments was again overcome by the use of a different set of baseline stimulation and tetanization parameters. The parameters used were a modified version of those routinely used with success by Errington and colleagues (e.g. Errington et al., 1995). Baseline test intensity was set, as before, at an intensity sufficient to elicit a small population spike (2-4 mV), but pulse width was set at 50 μs, rather than 100 μs as before, and monophasic rather than biphasic pulses were delivered. During tetanization, the pulse width was doubled to 0.1 mA, but the stimulation intensity was not changed. The tetanus consisted of 3 trains of 50 pulses at 250 Hz. In previous studies using this tetanus, an inter-train interval of 1 min has typically been used. However, owing to the need to deliver low frequency stimulation very soon after tetanization in experiment 8.2, pilots were carried out with an interval of 20 s, and a 10 s inter-train interval was finally adopted.

There are several differences between the parameters described above and those used in previous experiments, none of which were systematically investigated. For instance, monophasic stimulation was adopted purely in order to replicate the conditions described by Errington and colleagues. However, a number of other factors relating both to the new parameters are worth addressing, even if conclusive data are unavailable.

It has been suggested, though never systematically studied, that optimal LTP is induced by widely spaced tetanus trains. A potential mechanism for such an effect might be adenosine release during tetanization, a phenomenon that might block further potentiation induced by subsequent trains delivered in close succession (see Abraham and Huggett, 1997).

Figure 9.4.1 shows the amount of LTP induced by the tetanus described in the first paragraph of this section, with either a 20 s inter-train interval (n = 6) or a 10 s interval (n = 6). The latter data are

taken from the "tetanus only" control group of figure 8.2.2; The use of a 10 s inter-train interval did not result in a significantly different level of EPSP slope LTP after 1 hr compared to a 20 s inter-train interval [figure 9.4.1A; $F(1,10) = 2.44$; $p > 0.1$]. However, a slightly reduced level of EPSP slope PTP was seen in the 10 s condition [$F(1,10) = 7.35$; $p < 0.05$]. A trend towards a difference in the increase in population spike amplitude 1 hr after tetanization was also observed [figure 9.4.1B; $F(1,10) = 4.88$; $0.1 > p > 0.05$], and population spike PTP was significantly smaller in the 10 s condition [$F(1,10) = 3.69$; $0.1 > p > 0.05$]. These data reveal a slight effect of inter-train interval.

However, the main finding is that robust LTP was induced in both conditions, after a period of failure using previous tetanus parameters. Owing to the fact that the use of a 10 s inter-train interval resulted in successful LTP in the present study, but very little LTP in section 9.3.3, this factor cannot explain the failure of LTP induction.

It is worth noting that the tetanus intensity was fairly mild. Tetanic stimulation was delivered at an intensity of less than 0.3 mA, with a pulse width of 100 µs, i.e. approximately half the intensity used in experiment 7.3. Nevertheless, analysis of the first pulse of the first tetanus train in the "tetanus only" group of experiment 8.2 revealed that stimulation at this intensity evoked a mean EPSP slope of 5.39 ± 0.54 mV / ms, and a population spike amplitude of 7.65 ± 0.68 mV. It would be inappropriate to describe such stimulation as "weak". Nevertheless, in view of the detrimental effects of high tetanus intensities revealed in section 9.3.2, the use of a less intense tetanus may have contributed to the respectable levels of LTP displayed in figure 9.4.1.

9.4.3 The effect of baseline test pulse width on the level of LTP recorded

In experiment 8.2, baseline test pulse stimulation was delivered at a pulse width of 50 µs, half the value used in previous experiments. Halving the duration of the stimulus pulse is not equivalent to halving the current. For instance, a weak stimulating current would not be expected to activate as many afferent fibres as a strong current, regardless of the duration of the pulse.

Owing to the fact that the characteristics of the test pulse with which potentiation is sampled are critical in determining the level of LTP recorded (see section 9.3.2), pulse width I / O curves were recorded over a range of stimulus intensities both before and after tetanization. The following data were recorded from a single rat, a member of the 20 s inter-train interval group displayed in figure 9.4.1. During tetanization, pulses were delivered at 0.3 mA with a pulse width of 100 µs.

I / O curves were recorded both 20 min before and 1 hr after tetanization. Pulse widths ranged from 10 to 100 µs, and 4 different stimulation intensities were tested (0.1 mA, 0.2 mA, 0.4 mA, and 0.8 mA). Inspection of the raw data from the pre-tetanus I / O curve revealed that EPSP magnitude was not determined by a simple multiplication of stimulation intensity with pulse width (i.e. total charge

passed). The absolute EPSP slope elicited by a 0.8 mA test pulse of 10 μs duration was more than double the size of that elicited by a 0.1 mA test pulse of 100 μs duration (data not shown).

Figure 9.4.2 shows the amount of EPSP slope LTP at increasing pulse width over the 4 different stimulation intensities. Curves were constructed from the I / O data as described in experiment 7.3. The most striking finding is the EPSP slope depression at small pulse widths during 0.1 mA test pulse stimulation. Note that the scale has been extended in this panel. As in figures 9.3.3 & 9.3.4 (rat 3), the depression in EPSP slope was small in absolute terms, but large in percentage terms. At pulse widths lower than 50 μs, an EPSP slope of less than 1 mV / ms was elicited. The observed EPSP slope depression at pulse widths between 20-50 μs reflects an absolute decrease of 0.2 mV / ms or less in each case (data not shown).

As in figure 9.3.2, EPSP slope depression was only observed at a test pulse intensity of 0.1 mA. At a test intensity of 0.2 mA, EPSP slope depression was not observed at any pulse width, but potentiation was still greatest at high pulse widths. At 0.4 mA, an inverted U-shaped relationship between pulse width and LTP was obtained, and at 0.8 mA, potentiation was found to be greatest at small pulse widths, and declined as the pulse width was increased.

A scheme similar to that suggested in section 9.3.3 might also explain the present data. It is possible that potentiation of feed-forward inhibition overcomes the potentiation of excitatory transmission when test pulses of short duration are delivered at 0.1 mA. As the pulse width is increased, however, increases in excitation may begin to dominate. However, as stimulation intensity is increased still further, the effect of non-linear summation may become significant at long pulse durations, resulting in the inverted U-shaped relationship at 0.4 mA. At high intensities, potentiation of feed forward inhibition may no longer be a significant factor, but non-linear summation of postsynaptic potentials may become increasingly important, resulting in maximal potentiation at short pulse widths, and a gradual decline in LTP with increasing pulse width.

In conclusion, it seems that for this single rat at least, a pulse width of 50 μs was optimal over the range of baseline test pulse settings used in experiment 8.2 (approximately 0.2-0.5 mA). The LTP recorded with a pulse duration of 100 μs varied more as a function of stimulus current, being greater at low intensities, but smaller at high intensities. However, the fact that lasting LTP was recorded using test pulses of 100 μs in this example suggests that the difference in pulse width between experiment 8.2 and section 9.3.1 cannot be blamed for the failure of LTP in the latter study.

9.4.4 The effect of the strain of rat used on LTP

The effects of rat strain on synaptic plasticity have rarely been investigated. However, the use of Sprague-Dawley or Wistar rats is more common in LTP experiments than the use of Lister-hooded

rats. The use of Lister-hooded rats in this laboratory is due simply to that fact that these animals have good eyesight and are easy to track in the watermaze owing to the dark coloration of the head and neck. It has been reported that CA1 LTD is more readily obtained in freely moving Wistar rats than in Sprague-Dawley or Lister-hooded rats (Manahan-Vaughan and Reymann, 1997b). In fact, LTD was never obtained in the latter strain. It has also been found that Sprague-Dawley rats exhibit greater dentate PTP than Wistar-Kyoto rats (Diana et al., 1994). However, no differences in LTP were reported.

In order to test whether the use of a different strain of rat might provide a solution to the LTP problems experienced at the time when the experiment described in section 9.3.1 was carried out, a number of pilot experiments were conducted using a tetanus similar or identical to that used in experiment 7.3. Attempts were made to induce LTP in two Sprague-Dawley rats, and one male and one female Cob Wistar. In none of these cases was robust LTP induced (data not shown). Hence, although the influence of strain was not systematically investigated, the use of different strain did not provide an immediate solution to the problem.

In order to test the possibility that rats obtained from the pharmacology breeding colony were deficient in LTP, 4 Lister-hooded rats were obtained from a commercial supplier (Charles River U.K. Ltd.). However, these animals showed no more LTP than those bred in our own colony (see section 9.4.5).

A more extensive pilot study was not carried out owing to the finding, described in section 9.4.2, that a modified set of baseline and tetanization parameters resulted in robust LTP in Lister-hooded rats.

9.4.5 The effects of stress on LTP induction

It has been known for some time that stress can impair hippocampal LTP (e.g. Foy et al., 1987). Stress resulting from inescapable, rather than avoidable electric shock, appears to be particularly detrimental (Shors et al., 1989). In fact, merely exposing a rat to a novel environment can block the induction of LTP by a primed burst protocol (Diamond et al., 1990). It has recently been found that stress not only inhibits LTP, but also enables the induction of LTD *in vivo* (Xu et al., 1997). The effects of stress on synaptic plasticity are believed to be mediated largely by the release of glucocorticoids such as corticosterone (Filipini et al., 1991; Diamond et al., 1992; Pavlides et al., 1993; Xu et al., 1998a).

In all experiments from 7.4 onwards, rats were handled daily for approximately one week before LTP was measured. This was done in an attempt to reduce stress levels associated with handling during anaesthesia. However, before an experiment, rats were taken from the animal room to the surgery, then placed in a halothane chamber before being injected with urethane. This procedure probably

causes some degree of acute stress, owing to environmental novelty and the anaesthesia process itself. In order to overcome this potential problem 4 rats obtained from a commercial supplier (the same 4 Charles River rats mentioned in section 9.4.4) were habituated to the surgery environment for a period of 30 min over 8 consecutive days. This period included a brief daily spell in the anaesthetic chamber. When anaesthetized prior to electrophysiological recording, these rats showed no evidence of stress, such as vocalization or defecation. LTP was recorded as described in experiment 7.3. Tetanization parameters were varied slightly between rats, but were in all cases similar to those used in experiment 7.3. However, lasting EPSP slope potentiation was only induced in one rat, and even in this case the increase was a mere 10 % after 1 hr.

Although the above evidence is clearly inadequate to make substantial claims about the role of stress in LTP induction, it would seem that a simple habituation procedure does not provide an immediate solution during periods when LTP induction routinely fails. However, the process of anaesthesia itself may be stressful, and habituation to this procedure is clearly not feasible. Moreover, LTP is successfully induced in many laboratories without extensive prior handling of the rats, habituation to the surgical environment, or even halothane anaesthesia prior to intraperitoneal injection of anaesthetic. Hence, it seems unlikely that stress is the critical factor underlying the intermittent failure of LTP in this laboratory.

9.4.6 Seasonal variations in LTP

As mentioned above, problems with the induction of LTP tended to occur in the winter, rather than in the spring or summer. Owing to the variety of tetanus and baseline stimulation parameters used throughout the work described in this thesis, an overall analysis of seasonal effects is impossible. However, the tetanus used in experiment 8.2 produced robust LTP in June / July when the main experiment was conducted (figure 8.2.2; "tetanus only" control group), but resulted in modest LTP when tested in December (figure 8.4.1; "tetanus only" control group for 1 Hz experiment). The percentage EPSP slope LTP measured over the final 10 min of recording (50-60 min after tetanization) is shown in figure 9.4.3. The difference in LTP between June / July and December was highly significant [$F(1,10) = 18.8$; $p < 0.01$], although equivalent PTP over the 4 min after tetanization was observed [$F(1,10) = 1.30$; $p > 0.2$]. The population spike increase 1 hr after tetanization was slightly, but non-significantly, smaller in December than in June / July [3.87 ± 0.42 mV and 3.12 ± 0.65 mV respectively; $F < 1$]. No group differences in baseline stimulation intensity were found [$F(1,10) = 2.07$; $p > 0.1$; data not shown].

The existence of seasonal changes in synaptic plasticity is surprising considering the use of male rats housed from birth in a temperature-controlled environment with a fixed 12 hr light / 12 hr dark cycle. Nevertheless, pronounced seasonal cycles in the levels of hypothalamic neuropeptides, such as

corticotrophin releasing factor (CRF), have been reported in male Sprague-Dawley rats under similarly uniform conditions (Bisette et al., 1995). The application of CRF is known to increase the secretion of corticosterone (Yasuda et al., 1984), a hormone that has been found to inhibit LTP (see section 9.4.5). Hence, seasonal changes in the secretion of neuromodulatory agents such as corticosterone may provide a possible mechanism for apparent seasonal changes in LTP magnitude.

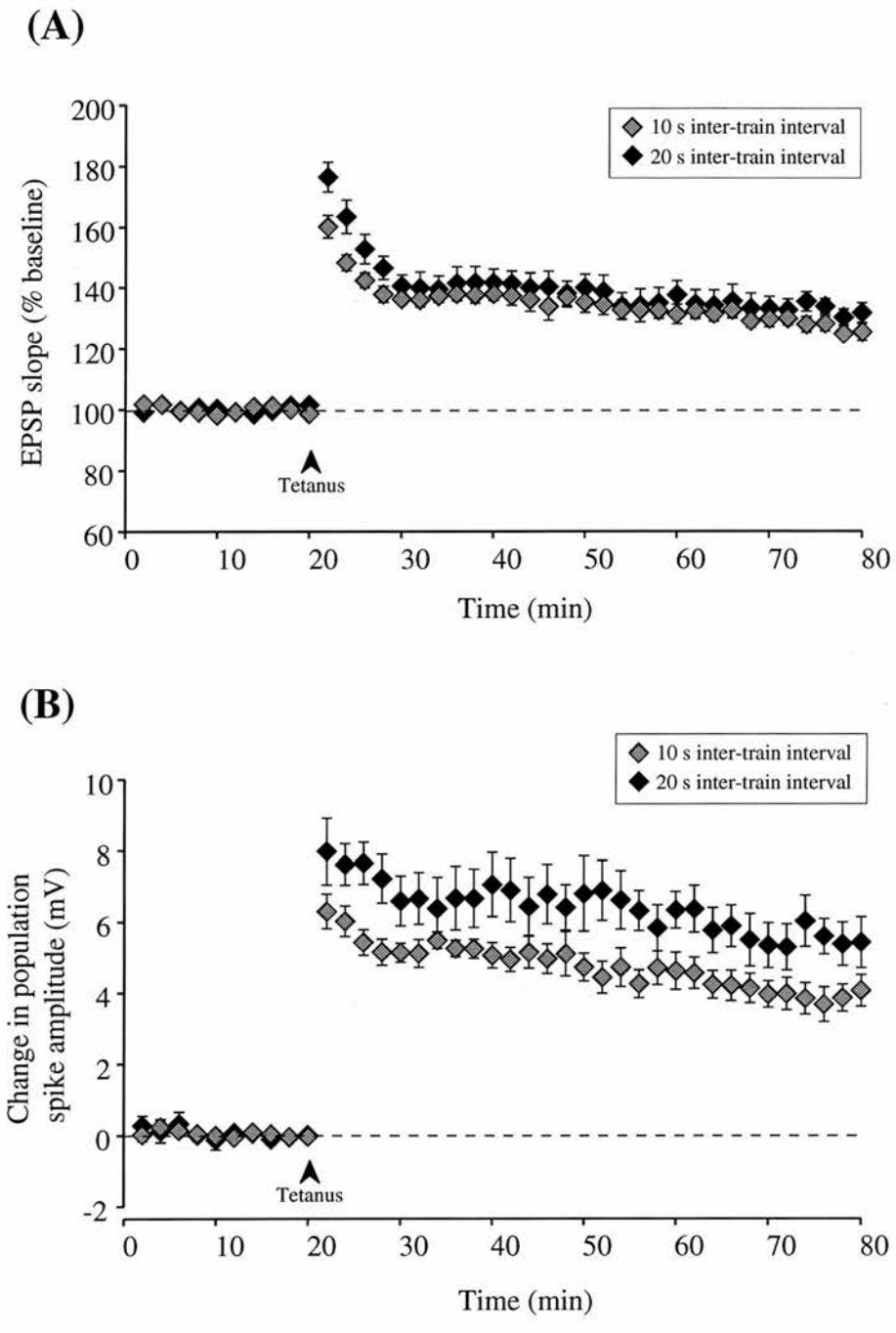


Fig. 9.4.1

Tetanization with 3 trains of 50 pulses at 250 Hz. An inter-train interval of either 10 s or 20 s was used (see text for further details).

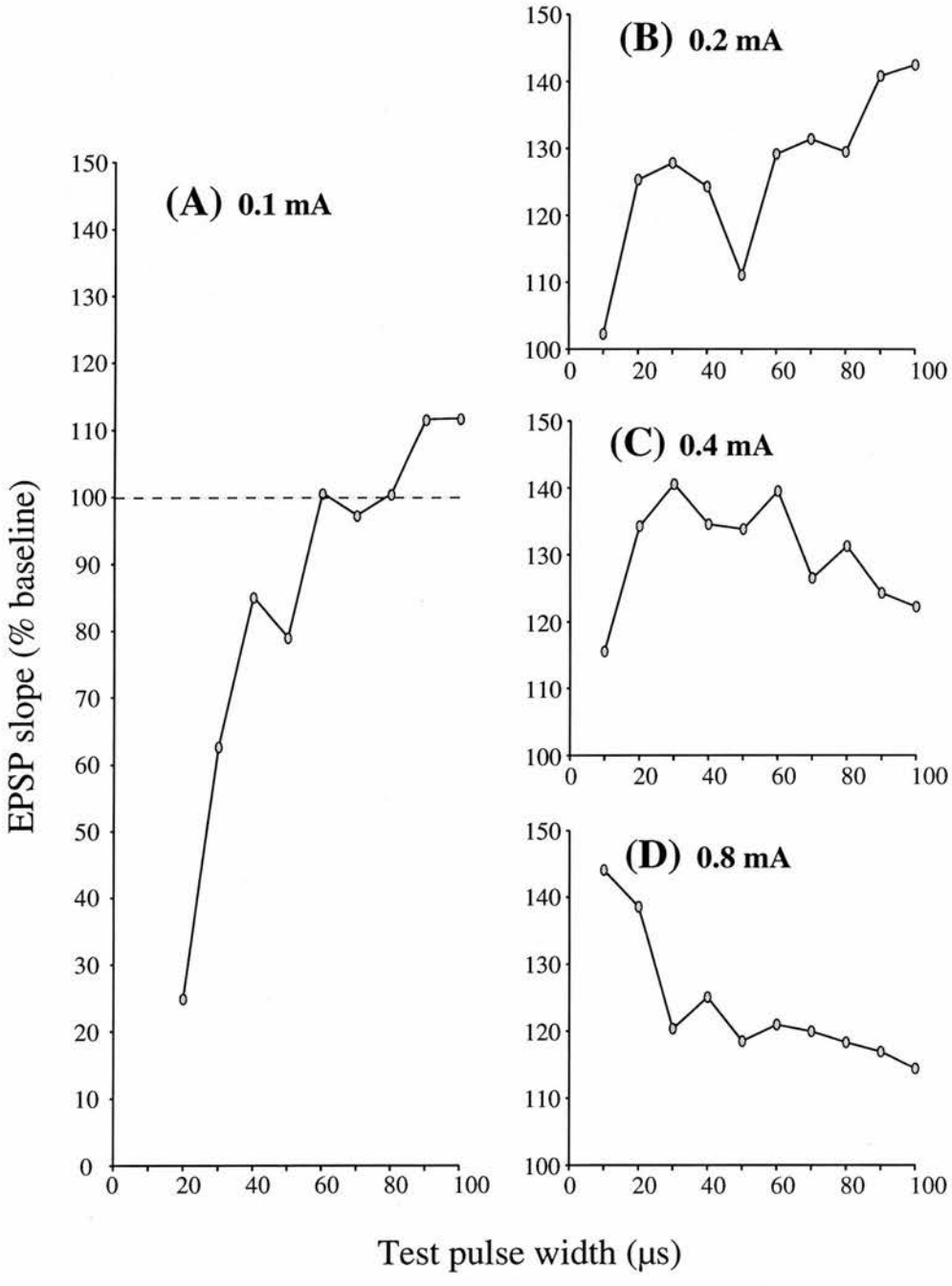


Fig. 9.4.2

The relationship between EPSP slope LTP and test pulse width at a range of different test pulse intensities indicated in the top left corner of each panel. Scaling is equivalent in each case, although the y-axis has been extended from 100 % down to 0 % in part (A).

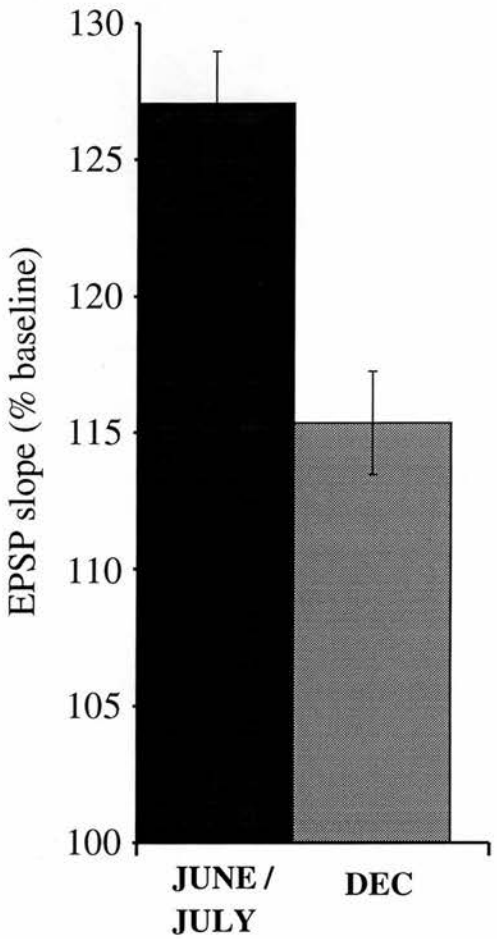


Fig. 9.4.3

LTP induced by an identical tetanus in summer and in winter.

9.5 General Discussion

The findings presented in this chapter represent an attempt to identify at least some of the factors associated with the magnitude of LTP. However, apart from the suggestion that seasonal hormonal rhythms might play some role, the reason for the intermittent failure of LTP induction remains unknown. Nevertheless, in the course of this analysis, some intriguing and counterintuitive results have been obtained.

Firstly, the size of an EPSP seems to be negatively correlated with the percentage EPSP slope LTP induced, at least under some circumstances. The suspicion that "good" EPSPs exhibit poor potentiation has been raised by others, but rarely investigated. Although by no means the only possible explanation, it is suggested that non-linear summation of postsynaptic potentials may underlie this paradoxical result.

Secondly, it was found that the use of a high intensity tetanus fails to induce robust potentiation during periods of LTP failure. In fact, tetanus intensity was negatively correlated with the magnitude of EPSP slope LTP over the range tested. This result is surprising, considering the well-established concept of cooperativity, namely that the amount of LTP can increase as a function of the number of perforant fibres activated during tetanization (McNaughton et al., 1978). The reasons for this discrepancy are unknown, but it is possible that the relationship reported by McNaughton and colleagues only holds over a limited range of tetanus and / or test pulse intensities.

A more general issue arising from the results of this chapter concerns the use of input / output curves. In many studies of LTP, potentiation is recorded at a single arbitrary test pulse intensity. Baseline test pulse characteristics such as current, duration and the use of monophasic vs. biphasic stimulation vary widely between laboratories, but are rarely recognized as critical factors in determining the level of LTP recorded. In fact, as revealed in sections 9.2 and 9.3, results ranging from substantial LTD to robust LTP can be observed in the same animal depending on the sampling intensity used. Owing to the widespread failure to characterize changes in synaptic efficacy over a range of test intensities, it is difficult or impossible to compare results obtained in different laboratories. It is possible that this issue contributes substantially to a number of conflicting reports in the LTP literature.

However, a potential drawback with the recording of I / O curves is the possibility that high intensity test pulses, delivered, at 0.1 Hz may themselves induce changes in synaptic efficacy. Indeed, a slight rise in baseline EPSP slope was sometimes observed after an initial I / O curve (data not shown). However, such changes were generally small, and hence unlikely to affect the level of LTP subsequently obtained. Nevertheless, in future it would be wise to deliver I / O curve test pulses at

0.05 Hz, and to randomize the order in which pulses of different intensities are presented, rather than simply increasing the intensity in stepwise fashion.

A further methodological dilemma is presented by the question of which attribute of an EPSP to use as an index of potentiation. Measurement of the population spike amplitude is favoured by some, because it represents a physiologically relevant output, i.e. the firing of cells. However, population spike amplitude reflects a confound of the number of cells firing, the degree of synchrony, and possible changes in excitability due to neuromodulators. Owing to this problem, the change in EPSP slope is more commonly used as a measure of LTP. It is generally believed that the early rising phase of the EPSP slope provides a good index of fast synaptic transmission. However, as illustrated in figures 9.3.1A and B, potentiation of population spike amplitude is not always accompanied by lasting EPSP slope LTP. In fact, it is sometimes observed that after tetanization, despite lasting changes in the amplitude and latency to onset of the population spike, and sometimes even changes in the amplitude and overall shape of the EPSP, the EPSP slope remains unchanged. This is not an argument for abandoning the use of EPSP slope as a measure of synaptic efficacy. The drawbacks associated with alternative measures are even more severe. However, it should be remembered that LTP is sometimes not manifested by a robust increase in EPSP slope.

One factor that has not been addressed in this chapter is anaesthesia. The possible role of urethane on LTP induction was discussed in chapter 7.7. However, pilot studies not included in this thesis have revealed that difficulties in the induction of LTP can be equally severe in freely moving as in urethane-anaesthetized rats. In spite of these problems, the issue of anaesthesia remains of particular relevance in studies of the role of mGluRs in LTP (see chapter 7.7).

To summarize, a number of factors have been identified which influence the amount of LTP recorded after tetanization, although many sources of variability remain unexplained. The overall conclusion of this investigation is that for anaesthetized rats in this laboratory, a test pulse stimulation intensity of 50 μs, at an intensity of 0.2-0.4 mA appears to be optimal. Tetanic stimulation should be delivered at a modest intensity, in order to avoid the EPSP slope depression associated with intense tetanization. Finally, LTP experiments should be carried out in summer.

Chapter Ten

Summary

10.1 Introduction

The initial aim of this thesis was to investigate the role of NMDA receptors and mGluRs in spatial learning. This led to a series of experiments investigating the role of mGluRs in LTP, followed by an electrophysiological study of the time dependency of LTP reversal by 5 Hz stimulation. This concluding section provides a brief summary of the experimental findings and their implications.

10.2 D-AP5 and spatial learning

The first experiment (chapter 5) was designed to address two main issues: (a) whether chronic minipump infusion of D-AP5 causes brain damage; and (b) the effects of a specific behavioural protocol described by Cain et al. (1996) on drug-induced sensorimotor disturbances. The results of this study reveal that chronic AP5 infusion does not cause a persistent spatial learning deficit after infusion has ended, such as that reported after MK-801 infusion in mice (Wozniak et al., 1996). In addition, although acute intracerebral infusion of AP5 causes neuronal vacuolation (Labruyere et al., 1989) no signs of neurotoxicity were observed in brains removed either during or after slow, chronic AP5 infusion. These results provide convincing evidence that AP5-induced neuronal damage is not the cause of the learning impairment resulting from infusion of this drug.

The sensorimotor disturbances caused by AP5 were mild at the start of training, but became progressively more severe as testing progressed. Under these circumstances, the nature of the performance deficit is impossible to assess. The massed-trial protocol of Cain et al. (1996) greatly exacerbated the sensorimotor side effects of AP5 infusion, compared to those seen with 1 trial per day training (Bannerman et al., 1995). It has been suggested that sensorimotor deficits are the cause of the failure to learn a watermaze task after the delivery of AP5. However, it is also possible that an initial AP5-induced failure to learn results in a progressive decline in sensorimotor aspects of performance when coupled with multiple training trials in a task which for drug-treated animals is insoluble.

The results of the above study do not provide definitive evidence that the AP5-induced impairment results from a specific learning deficit. Nevertheless, a subsequent series of experiments carried out in this laboratory by other members of the research group has provided positive evidence in support of this notion. Rats were trained in a spatial discrimination task in the watermaze, in which a stable

platform was placed in a fixed location, but an unstable platform was placed in a random location. This study revealed that whereas AP5-induced sensorimotor deficits and abnormal behaviours declined to control levels over the first few days of testing, discrimination performance remained impaired in AP5-treated rats (R. G. M. Morris, R. J. Steele, S. J. Martin, and J. E. Bell, unpublished observations). This indicates that the AP5-induced learning impairment is not inextricably linked with the sensorimotor deficit. In addition, it has recently been found that AP5 causes a delay-dependent impairment in a watermaze matching-to-place task, a result that is difficult to explain as anything but a learning impairment (Steele and Morris, 1999). Hence, the balance of current evidence suggests that AP5 does indeed cause a learning deficit in certain spatial tasks. Although AP5 infusion does cause various non-mnemonic deficits, these cannot account for the poor performance of rats in all learning and memory tasks.

10.3 The effects of MCPG on LTP and spatial learning

Few definitive conclusions can be drawn from the effects of the mGluR antagonist MCPG on watermaze performance described in chapter 6. In the first experiment of this chapter, acute i.c.v. infusion of MCPG caused a watermaze deficit that was limited to the transfer test, although a slight initial deficit was also seen in a visible platform task. However, subsequent experiments revealed that MCPG had no effect when chronically administered via a minipump, or when injected acutely into the hippocampus. In a final study, there was a suggestion that diffusion of MCPG beyond the hippocampus might be associated with a behavioural deficit. Whilst not ruling out the possibility of a learning deficit specific to the blockade of hippocampal mGluRs, a non-hippocampal sensorimotor or motivational account might also explain these data. Furthermore, it is possible that MCPG may be too weak an mGluR antagonist to induce a reliable behavioural deficit, regardless of the nature of this impairment. In addition, the subtype selectivity of MCPG is broad, and may include as yet unidentified mGluRs. In future, it would be interesting to test the effects of a more potent and subtype specific mGluR antagonist using a more powerful behavioural paradigm, such as the matching-to-place task used by Steele and Morris (1999). As mentioned in chapter 6, pilot studies using AIDA are currently underway.

MCPG had no effect on dentate LTP under urethane anaesthesia *in vivo* (chapter 7). This was true for all experimental conditions, even when MCPG was applied after the reversal of prior LTP. Although this last result is apparently inconsistent with the "molecular switch" hypothesis of Bortolotto et al. (1994), there is reason to believe that the mechanism of LTP reversal by 5 Hz stimulation delivered 2

min after tetanization may be different from the reversal of established LTP (see chapter 8.5.3). Hence, no strong conclusions about the validity of the molecular switch hypothesis can be drawn from the present data.

The MCPG studies presented in this thesis can therefore be summarized as follows: a drug that has no effect on LTP has only modest effects on learning. This series of results unfortunately reveals very little about the mechanistic relationship between LTP and learning, and the questionable efficacy of MCPG as an mGluR antagonist prevents any strong negative conclusions about the role of mGluRs in LTP and learning being made. However, a number of issues concerning the role of MCPG in LTP remain unexplored. Perhaps the most important of these is the question of anaesthesia. It has been reported that MCPG can completely block LTP in freely moving rats, whereas a partial block or no effect is generally reported under urethane (see chapter 7.7). One possible explanation is that weaker tetanization parameters may be sufficient to induce LTP *in vivo* (Riedel et al., 1994b). It has recently been reported that MCPG is only effective in blocking the LTP induced by a weak tetanus (Wilsch et al., 1998). However, pilot studies not included in this thesis have revealed that the induction of LTP in freely moving rats tends to be even more problematic than the induction of LTP under urethane (see chapter 9). Although, so far, it has not been possible to test the effect of MCPG on the LTP induced by a weak tetanus *in vivo*, it is hoped that such an experiment may be carried out in the near future.

10.4 Time-dependent reversal of LTP

It was reported in experiment 7.4 that low frequency stimulation could reverse dentate LTP *in vivo* when delivered soon after tetanization. In chapter 8, a detailed characterization of the time-dependent reversal of dentate LTP by 5 Hz stimulation was presented. It was found that 5 Hz stimulation was only successful in reversing LTP when delivered within 2 min of tetanization. 5 Hz stimulation had no effect on existing LTP when delivered 10 min or more after tetanization. It is worth noting that this form of "depotentiation" may be very different from the reversal of established LTP. For reasons discussed in chapter 8.5.3, it is likely that the present results represent a disruption of processes involved in the stabilization of LTP, rather than a genuine depotentiation of existing LTP.

The investigation of LTP reversal was motivated by the possibility of using the phenomenon as a tool in future behavioural experiments. The design of such an experiment is outlined in chapter 8.5.4. However, there are a number of grounds for caution. In particular, 5 Hz stimulation alone was associated with an E-S depression, consistent with a reduction in granule cell excitability.

Furthermore, seizure-like afterdischarge activity was always associated with 5 Hz stimulation, a phenomenon that is clearly undesirable and might constitute a serious problem in freely moving rats. Nevertheless, if this problem can be overcome, it will be worthwhile conducting a pilot study to assess the possible selective reversal of recent learning by the delivery of 5 Hz stimulation.

10.5 Conclusion

The role of NMDA receptors in LTP is well established, and the balance of current evidence suggests that NMDA receptor activation is also necessary for spatial learning. The results of chapter 5 reveal that the chronic application of AP5 is not neurotoxic, and that sensorimotor disturbances are not an inevitable consequence of AP5 infusion. A subsequent study carried out by Steele and Morris (1999) has revealed that AP5 infusion causes a delay-dependent deficit in watermaze matching-to-place performance. This finding cannot be attributed to sensorimotor disturbances, and is difficult to interpret as anything other than a memory impairment. Such a result, although not providing definitive proof, is highly suggestive of a link between NMDA receptor-dependent synaptic plasticity and spatial learning.

The role of mGluRs in both LTP and spatial learning is less clear, however. The antagonism of mGluRs has been reported to block LTP under some circumstances, but not others (see chapter 7). This result may simply reflect the inadequacy of MCPG as an mGluR antagonist, but may also indicate that mGluRs play a more subtle modulatory role in LTP than NMDA receptors. If so, then the role of mGluR activation in naturally occurring synaptic plasticity, if such a thing exists, may not be obligatory under all circumstances. Considering the difficulty in establishing a link between NMDA receptor activation and learning, it may be even harder to establish such a link for a modulatory class of receptor. Unfortunately, this gap between levels of analysis is only likely to be bridged once a "circuit-level" understanding of hippocampal function is reached.

In the meantime, progress will require the thorough characterization of novel and subtype-selective mGluR agonists and antagonists. However, the different mGluR subtypes have distinct physiological actions, and it may not be sufficient simply to demonstrate an impairment or facilitation of spatial learning resulting from the application of a novel pharmacological agent. Ultimately it is likely that the development of new behavioural tasks will be necessary. An adequate task will need to provide a rich characterization of behaviour, sufficient to identify the precise role of mGluRs, and indeed NMDA receptors, in hippocampus-dependent learning.

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Appendix

Published work

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(*R*,*S*)-α-Methyl-4-carboxyphenylglycine (MCPG) Fails to Block Long-term Potentiation Under Urethane Anaesthesia *in vivo*

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Summary—The effects of the metabotropic glutamate receptor antagonist (*R*,*S*)-α-methyl-4-carboxyphenylglycine (MCPG) on the induction of long-term potentiation (LTP) in the dentate gyrus were examined under urethane anaesthesia *in vivo*. In experiment 1, bilateral intraventricular infusion of either 20 mM or 200 mM (*R*,*S*)-MCPG (5 μl each side) failed to block LTP in the perforant path–granule cell projection, relative to vehicle-infused controls; 30 mM D-AP5 (5 μl each side) infused in the same way as MCPG completely blocked LTP. Experiment 2, in which the contralateral perforant path–dentate gyrus pathway was used as a non-tetanized control, revealed that slight baseline changes induced by MCPG infusion were transient; again no block of LTP was obtained. The efficacy of mGluR blockade was confirmed in experiment 3, in which MCPG antagonized an increase in spontaneous activity induced by (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD). In experiment 4, significant depotentiation was induced by low frequency stimulation (5 Hz for 1 min) given 2 min after high frequency tetanization, but MCPG remained ineffective in blocking LTP after a second tetanus. In experiment 5, increasing the period of low frequency stimulation from 1 to 10 min produced greater depotentiation, but still did not unmask an MCPG-sensitive component of LTP. These experiments fail to support a role for mGluRs in the induction of LTP in the dentate gyrus under urethane anaesthesia *in vivo*, nor do they support the idea that a metabotropic switch controlling sensitivity to MCPG is reset by depotentiation.

Keywords—Hippocampus, MCPG, long-term potentiation (LTP), depotentiation, metabotropic glutamate receptors.

Long-term potentiation (LTP) is a form of activity-dependent synaptic plasticity which is widely cited as a model of the cellular mechanisms underlying memory formation (Bliss and Lømo, 1973; Morris *et al.*, 1990; Bliss and Collingridge, 1993). It is well established that NMDA receptor activation is necessary for the induction of LTP in the dentate gyrus and area CA1 of the rodent hippocampus since potentiation is completely blocked, both *in vivo* and *in vitro*, by application of the selective NMDA antagonist AP5 (Collingridge *et al.*, 1983; Harris *et al.*, 1984; Morris *et al.*, 1986; Errington *et al.*, 1987). However, direct iontophoresis of NMDA in hippocampal slice preparations induces only a short-lasting potentiation which decays back to baseline values within about 30 min (Collingridge *et al.*, 1983; Kauer *et al.*, 1988). In contrast, potentiation induced by a high frequency tetanus consists of a short-lasting decremental phase similar to that induced by NMDA, followed by a stable increase in synaptic efficacy which may remain above baseline values for hours or even days. It has been suggested that activation of metabotropic glutamate receptors (mGluRs) may provide the necessary additional trigger for the induction of stable NMDA receptor-dependent LTP (Behnisch and Reymann, 1993).

The metabotropic glutamate receptor family currently consists of eight subtypes (mGluRs 1–8) grouped into three sub-classes based on sequence homology, pharmacology and second messenger coupling. Class I comprises mGluRs 1 and 5 which stimulate phosphoinositide (PI) hydrolysis and intracellular calcium release. Class II (mGluRs 2 and 3) and class III (mGluRs 4, 6, 7 and 8) are negatively coupled to adenylate cyclase, but their pharmacological profiles differ (Nakanishi, 1992; Pin and Duvoisin, 1995). The most commonly used mGluR agonist is (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) which stimulates both class I and class II receptors; class III receptors are relatively insensitive to ACPD. Early studies revealed that the magnitude of tetanus-induced LTP in area CA1 *in vitro* is

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greatly increased by prior application of *trans*-ACPD, a mixture of (1*S*,3*R*) and (1*R*,3*S*)-ACPD (McGuinness *et al.*, 1991; Otani and Ben-Ari, 1991). It has since been suggested that activation of mGluRs may in itself be sufficient to induce a form of long-term potentiation. Bortolotto and Collingridge (1993, 1995) reported that the application of (1*S*,3*R*)-ACPD *in vitro* can induce a slow-onset potentiation in area CA1 which occludes totally with tetanus-induced LTP. However, Davis and Laroche (1996) found that whereas (1*S*,3*R*)-ACPD infusion *in vivo* caused a slow-onset potentiation in area CA1, a dose-dependent biphasic effect was obtained in the dentate gyrus, consisting of a short-term potentiation induced by both 10 mM and 25 mM ACPD, followed in the 25 mM group only, by a lasting depression of the response. Since ACPD is a broad-spectrum agonist acting at both class I and class II mGluR subtypes, it is possible that the potentiation of the EPSP is mediated by class I mGluRs coupled to PI hydrolysis, whereas the depression is mediated by presynaptically located class II mGluRs. The differential effects of ACPD in the dentate versus CA1 may reflect the different mGluR subtype populations within these two regions (Fotuhi *et al.*, 1994).

Early studies of the role of mGluRs in LTP were hampered by the lack of specific and potent mGluR antagonists. However, since its discovery, the selective mGluR antagonist (*R*,*S*)-α-methyl-4-carboxyphenylglycine (MCPG) has been widely used (see Watkins and Collingridge, 1994). MCPG, like ACPD, has been reported to act on both class I and II mGluRs (see Discussion). Nevertheless, studies of the effects of mGluR antagonists on the induction of LTP have often yielded contradictory results. For instance, Bashir *et al.* (1993) found that application of (*R*,*S*)-MCPG blocked LTP in rat CA1 hippocampal slices, but others have failed to replicate this result (Chinestra *et al.*, 1993; Manzoni *et al.*, 1994). MCPG has also been reported to block LTP in the dentate gyrus *in vivo*. Riedel *et al.* (1995) reported a complete block of LTP in freely-moving rats, and Richter-Levin *et al.* (1994) reported a reduction in EPSP slope potentiation following i.c.v. infusion of MCPG under urethane anaesthesia. However, other workers have found no effect of MCPG on LTP in urethane-anaesthetized animals (Bordi and Ugolini, 1995).

One potential explanation for these mixed results is provided by the findings of Bortolotto *et al.* (1994) who reported that in area CA1 *in vitro*, MCPG failed to block LTP in pathways which had previously undergone potentiation. However, low-frequency depotentiating stimulation was found to re-establish the ability of MCPG to block LTP following a second tetanus. These data suggest that the activation of mGluRs turns on a "molecular switch" which eliminates the requirement for further mGluR activation in the induction of LTP. Similarly, Wang *et al.* (1995) found that a block of LTP with MCPG was attained only after low frequency stimulation of afferent fibres. Negative results have, however, been reported by Selig *et al.* (1995) and Thomas and O'Dell (1995) who failed to see a block of LTP in the presence of MCPG even after low frequency stimulation of afferents. Nevertheless, the molecular switch hypothesis has not, to our knowledge, been tested *in vivo*. If, for unknown reasons, the switch is tonically activated *in vivo*, blockade of LTP with MCPG might become apparent only after low frequency stimulation has induced depotentiation and so reset the switch.

The following series of experiments was carried out to determine, initially, whether or not in our hands, MCPG blocks LTP and reverses the electrophysiological effects of ACPD under urethane anaesthesia *in vivo*. Additional experiments were then conducted in order to address the "molecular switch" hypothesis outlined above. However, rather than simply giving low frequency stimulation followed by MCPG infusion and LTP induction, it was decided to follow the Bortolotto *et al.* (1994) protocol in which LTP is first induced, then depotentiated using low frequency stimulation prior to drug infusion and further tetanization.

METHODS

Animals

Adult male Lister hooded rats (250–500 g) were used as subjects. They were given *ad libitum* access to food and water and were maintained on a 12 hr light–12 hr dark cycle.

Electrophysiology

Experiments were conducted under urethane anaesthesia (1.5 g/kg i.p.), with the rat mounted in a stereotaxic frame (Kopf, Tujunga, CA, U.S.A.) with skull horizontal. The animal's temperature was monitored by a rectal probe and maintained at 36.2 ± 0.2°C. Teflon-coated stainless steel electrodes were lowered into one or both hippocampal formations in order to record positive-going field potentials. A bipolar stimulating electrode was positioned in the angular bundle of the perforant path (AP = −7.5 mm, Lat = 4.0 mm) and a monopolar recording electrode in the hilus of the dentate gyrus (AP = −4.0 mm, Lat = 2.0 mm). Two stainless steel injection cannulae, connected by plastic tubing to Hamilton syringes, were lowered into the left and right lateral ventricles (AP = −0.9 mm, Lat = ±1.3 mm) and in experiments 1–3, a thermistor was lowered into the right dentate gyrus to monitor brain temperature. Field EPSPs and thermistor readings were amplified using a polygraph (Grass Instruments, Quincy, MA, U.S.A.); the initial slope of the field EPSP (measured by linear regression between two fixed time points) and the population spike amplitude were monitored on-line by an Acorn A5000 computer running specialized software.

Following the implantation of electrodes, stimulation was turned off for 30 min to allow the brain tissue to settle. After this period, a number of test pulses were delivered and the stimulation intensity was adjusted to

elicit a population spike amplitude of 2–4 mV. Recording was started as soon as stable potentials had been obtained. Throughout an experiment, perforant path test stimulation consisted of biphasic pulses of 100 μs half-pulse duration delivered at 0.05 Hz. Tetanic stimulation consisted of five trains of 20 pulses at 250 Hz, with 10 sec between trains; the stimulation intensity was set at twice the baseline level.

Drugs

Two different concentrations of (*R,S*)-MCPG (Tocris, Bristol, U.K.) were tested: 20 mM and 200 mM. Solutions were prepared by first dissolving the solid, crystalline form of (*R,S*)-MCPG in 1 M sodium hydroxide solution to form an equimolar solution, then diluting with phosphate buffered saline to achieve the desired concentration. Despite the high concentration of NaOH present, the pH of the 200 mM solution was found to be 7.28, i.e very nearly neutral. Two concentrations of (1*S*,3*R*)-ACPD (1 mM and 4 mM; Tocris) were prepared in the same way. A 100 mM stock solution of D-2-amino-5-phosphopentanoate (D-AP5; Tocris) was made up in equimolar NaOH. Spiking with small aliquots (1–2 μl) of 5 M NaOH was sometimes required to ensure that the drug dissolved fully. A final concentration of 30 mM D-AP5 was attained by dilution with the appropriate volume of aCSF. Solutions were stored at −20°C in small aliquots before use.

Statistics

All statistical comparisons were carried out using analysis of variance (ANOVA) unless otherwise stated. In general, statistical descriptions of the data are given in the main body of the text, whereas numerical values are included in the figure legends; such values are stated as mean $\pm$SEM throughout.

Examples of the actual initial values of a number of measures such as EPSP slope, population spike amplitude and brain temperature are given in the Results section of experiment 1.

RESULTS

Experiment 1: does MCPG block dentate LTP in vivo?

A 20-min baseline period was recorded, followed by a 10 μl bilateral drug infusion (i.e. 5 μl per ventricle) of either 200 mM (*R,S*)-MCPG ($n = 7$), 20 mM (*R,S*)-MCPG ($n = 6$) or vehicle (phosphate buffered saline (PBS); $n = 7$), delivered over a period of 10 min. Half an hour after the end of this infusion, animals received a high frequency tetanus. Field potentials were recorded for a further 2 hr after tetanization. In order to test the drug infusion system, a further control group was infused with 30 mM D-AP5 ($n = 6$). In two of these AP5-infused rats, recordings were made bilaterally; only one pathway received a high frequency tetanus.

EPSP slope LTP. Figure 1(A) shows the amount of EPSP slope potentiation in the three main groups, normalized to the mean value obtained during the 10 min prior to tetanization; neither dose of MCPG caused a block of LTP. Separate ANOVAs of the mean potentiation over different time periods revealed no significant group differences in EPSP slope LTP 50–60 min post-tetanus [$F < 1$] or 110–120 min post-tetanus [$F(2,17) = 1.21$; $p > 0.3$]. No group differences were found in the percentage post-tetanic potentiation (PTP) recorded 0–4 min after tetanization [$F < 1$]. The mean absolute slope of the pre-infusion baseline field EPSP did not differ across groups [200 mM MCPG = 3.48 $\pm$ 0.37 mV/msec; 20 mM MCPG = 3.61 $\pm$ 0.28 mV/msec; vehicle = 3.61 $\pm$ 0.33 mV/msec; $F < 1$], nor did the test-pulse stimulation intensity [200 mM MCPG = 282.9 $\pm$ 39.7 μA; 20 mM MCPG = 291.7 $\pm$ 24.1 μA; vehicle = 318.6 $\pm$ 39.8 μA; $F < 1$].

Infusion of 30 mM D-AP5 caused a complete block of LTP. Figure 1(B) shows pooled data from the six rats infused with AP5 compared with the vehicle-infused controls. Mean non-tetanized control pathway data from the two rats in which recordings were made bilaterally are plotted alongside data from the six tetanized pathways. Curiously, infusion of AP5 caused a slight increase in EPSP slope, an effect noticeable in both tetanized and control pathways and associated with a transient temperature rise of approximately 0.5°C (data not shown). However, the elevated pre-tetanus slope magnitude appears to be maintained throughout the experiment. Inspection of Fig. 1(B) suggests that this is most likely due to the superposition of an EPSP slope increase induced by AP5, upon a slight chronic baseline rise. (A similar slight baseline rise was found in later experiments: see Fig. 2(A), control pathways.) Nevertheless, equivalent changes were seen in both tetanized and non-tetanized pathways. In the two rats which received bilateral stimulation and recording, no differences were found between tetanized and non-tetanized EPSP slope values 1 hr after tetanization, relative to the pre-tetanus baseline, indicating that LTP was indeed completely blocked (individual traces not shown). Hence, in these two animals at least, the possibility that a residual potentiation is masked by baseline changes can be ruled out.

Population spike LTP. Figure 1(C) shows the population spike potentiation expressed as the absolute change in amplitude relative to the mean baseline value over the 10 min prior to tetanization. This method of analysis results in a measure of change which is independent of initial baseline values. (A percentage measure of potentiation can result in misleading results if baseline values fall to low levels prior to tetanization, as occasionally happened.) No significant group differences were found 50–60 min post-tetanus [$F < 1$] or 110–120 min post-tetanus [$F < 1$]. No group differences were found in PTP 0–4 min post-tetanus [$F < 1$]. The mean absolute pre-infusion population spike amplitude did not differ across groups [200 mM MCPG = 2.46 $\pm$ 0.18 mV;

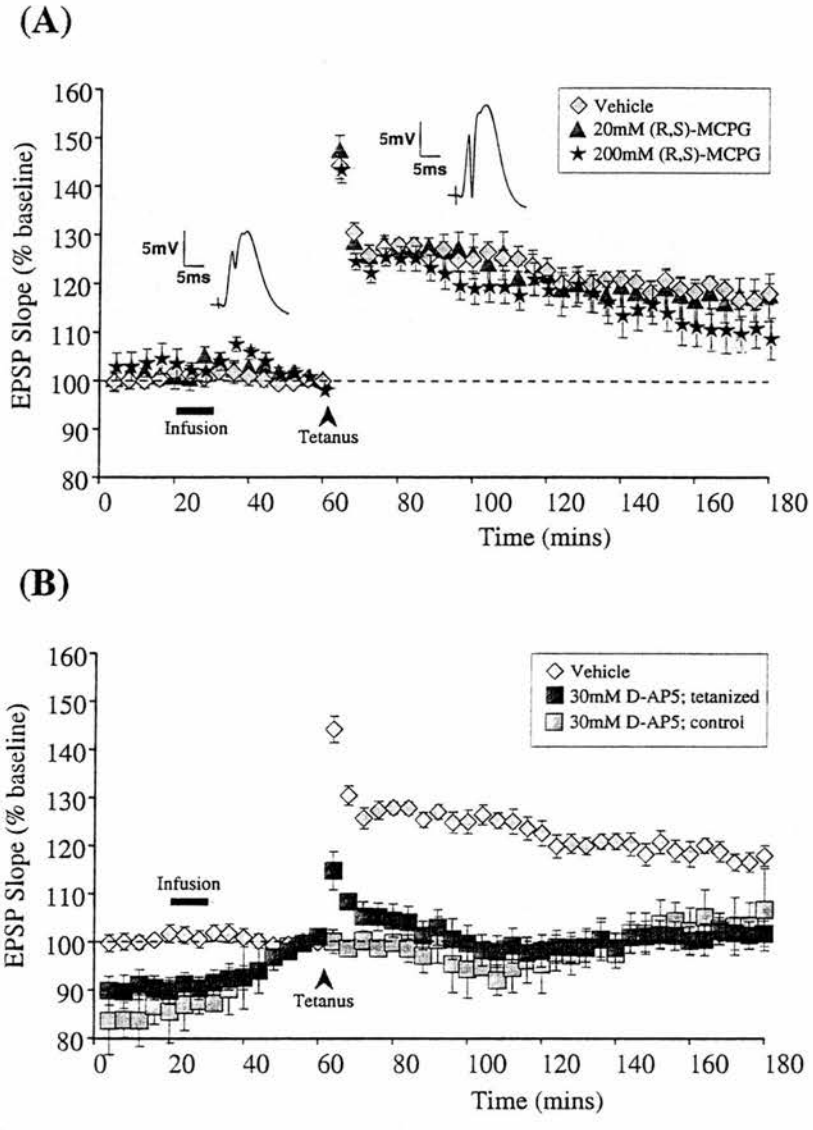

Fig. 1. Single pathway study. (A) Intraventricular infusion (10 μl) of either 20 mM ($n = 6$) or 200 mM (*R,S*)-MCPG ($n = 7$) had no effect on the percentage EPSP slope potentiation induced by a high frequency tetanus delivered to the perforant path–dentate gyrus projection, relative to vehicle-infused controls ($n = 7$). Mean percentage slope changes are plotted for each group, normalized to the baseline value over the 10 min prior to tetanization. Representative sample waveforms recorded immediately before tetanization and 30 min afterwards are shown. No group differences were found 50–60 min post tetanus [200 mM (*R,S*)-MCPG = 119.9 $\pm$ 3.2%; 20 mM (*R,S*)-MCPG = 121.8 $\pm$ 3.5%; vehicle = 123.3 $\pm$ 2.4%] or 110–120 min post-tetanus [200 mM (*R,S*)-MCPG = 109.9 $\pm$ 4.0%; 20 mM (*R,S*)-MCPG = 116.3 $\pm$ 4.6%; vehicle = 116.9 $\pm$ 2.4%]. (B) Infusion of 10 μl 30 mM D-AP5 caused a complete block of EPSP slope LTP. Data from six animals infused with D-AP5 are plotted against the vehicle-infused control data from the previous figure. Of the six rats infused with D-AP5, two received bilateral stimulation and recording; mean data from the two control pathways are plotted alongside data from the six tetanized pathways. 16.9 $\pm$ 2.4% LTP was seen in the vehicle-infused group 1 hr after tetanization, compared to 1.7 $\pm$ 2.8% in the D-AP5 tetanized group. A 3.9 $\pm$ 5.5% mean increase was seen in the 2 D-AP5 control pathways. (C) Mean absolute increases in population spike amplitude relative to baseline levels are plotted. An equivalent increase was seen in all groups 50–60 min post-tetanus [200 mM (*R,S*)-MCPG = 3.10 $\pm$ 0.29 mV; 20 mM (*R,S*)-MCPG = 3.46 $\pm$ 0.36 mV; vehicle = 3.60 $\pm$ 0.76 mV] and 110–120 mins post tetanus [200 mM (*R,S*)-MCPG = 1.81 $\pm$ 0.48 mV; 20 mM (*R,S*)-MCPG = 2.25 $\pm$ 0.48 mV; vehicle = 2.51 $\pm$ 0.58 mV]. (D) Infusion of both 20 mM and 200 mM (*R,S*)-MCPG caused a slight transient temperature rise relative to vehicle-infused controls. The mean increase over the 30 min after drug infusion was calculated relative to the mean baseline temperature over the 10 min before infusion [200 mM (*R,S*)-MCPG = 0.30 $\pm$ 0.04°C; 20 mM (*R,S*)-MCPG = 0.47 $\pm$ 0.14°C; vehicle = 0.01 $\pm$ 0.09°C].

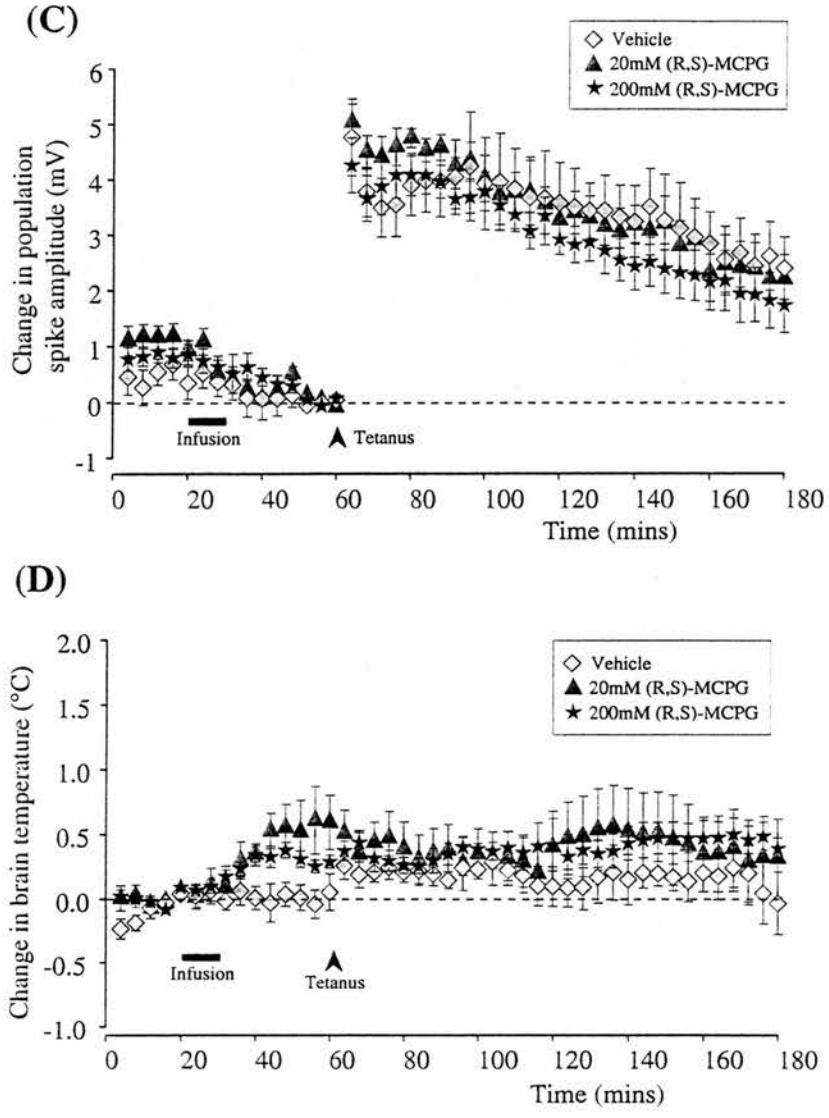

Fig. 1. Continued

20 mM MCPG = 2.92 ± 0.23 mV; vehicle = 2.51 ± 0.20 mV; $F(2,17) = 1.55$; $p > 0.2$].

Brain temperature. Figure 1(D) shows brain temperature changes throughout the experiment normalized to the mean temperature over the 10 min before infusion. An ANOVA of the mean temperature change over the 30 min after drug infusion revealed a significant effect of group [$F(2,17) = 6.04$; $p < 0.05$]. Individual Newman–Keuls pairwise comparisons revealed small but significant temperature increases in both the 20 mM MCPG ($p < 0.01$) and 200 mM MCPG groups ($p < 0.05$). These increases rarely exceeded 0.5°C, and no significant group differences were found over the final 30 min of the experiment [$F < 1$].

No significant differences were found in the absolute baseline brain temperature recorded over the 10 min prior to drug infusion [200 mM MCPG = 34.2 ± 0.4°C; 20 mM MCPG = 33.6 ± 0.2°C; vehicle = 32.5 ± 0.8°C; $F(2,17) = 2.80$; $p > 0.05$] The non-significant trend towards a group difference is explained by the slightly lower initial temperature recorded in the vehicle group: this is an artefact due to the use of a slightly different design of thermistor in three members of this group.

Experiment 2: does MCPG block LTP in vivo? A bilateral study

Most procedures were identical to those described for experiment 1. However, stimulating and recording electrodes were placed in the right hippocampal formation in addition to the left, and the thermistor was positioned approximately 2 mm posterior and 1 mm lateral to the right-hand recording electrode. The two pathways were stimulated alternately, but only the left perforant path was given a high frequency tetanus. Rats received infusions of either 200 mM (*R,S*)-MCPG ($n = 5$) or vehicle ($n = 7$). Data from three animals in which a seizure had occurred in one pathway during positioning of the electrodes were discarded. Likewise, if the final

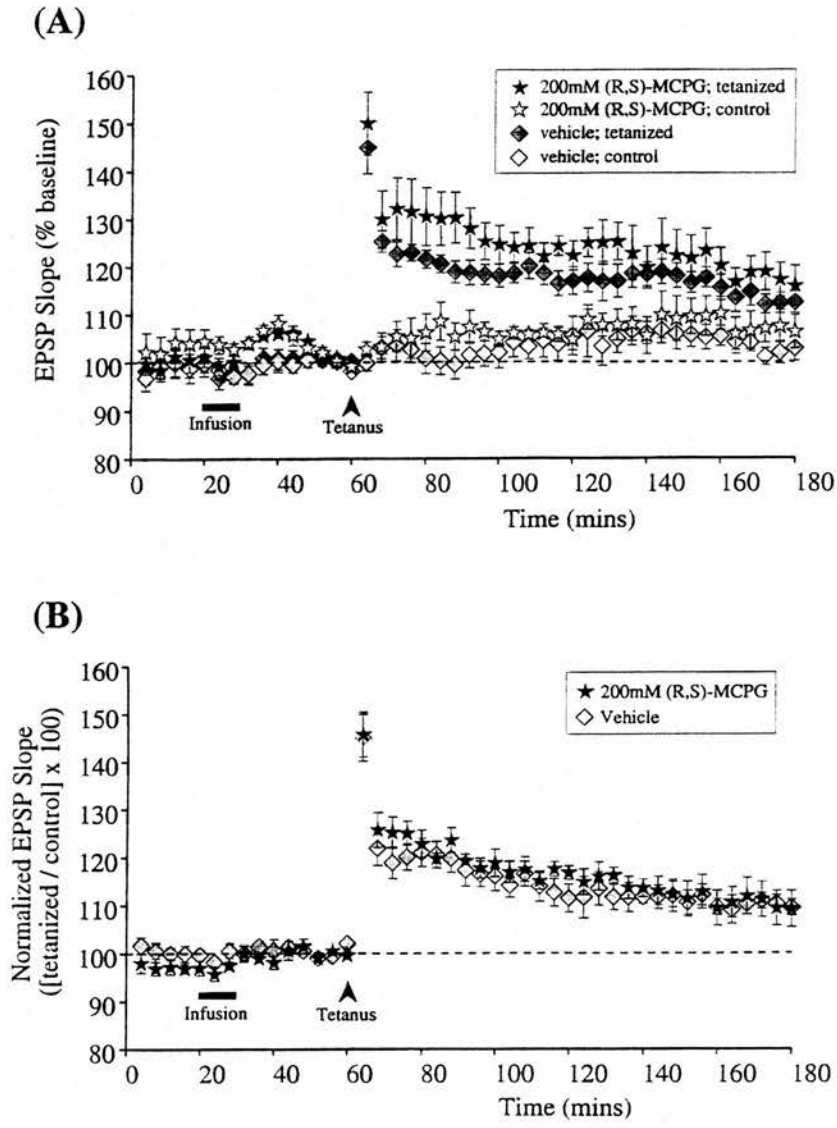

Fig. 2. Dual pathway study. (A) Intracerebroventricular infusion of 10 μl 200 mM (*R,S*)-MCPG ($n = 5$) failed to block EPSP slope LTP relative to vehicle-infused controls ($n = 7$). Tetanized pathways in each group showed equivalent LTP 50–60 min post-tetanus [200 mM (*R,S*)-MCPG = 122.8 $\pm$ 2.6%; vehicle = 117.0 $\pm$ 2.5%] and 110–120 min post-tetanus [200 mM (*R,S*)-MCPG = 116.7 $\pm$ 3.7%; vehicle = 112.5 $\pm$ 2.1%]. No group differences were found in the untetanized control pathways 50–60 min post-tetanus [200 mM (*R,S*)-MCPG = 105.4 $\pm$ 1.5%; vehicle = 104.3 $\pm$ 4.1%] or 110–120 min post-tetanus [200 mM (*R,S*)-MCPG = 106.5 $\pm$ 3.1%; vehicle = 102.2 $\pm$ 2.7%], indicating that despite the slight changes immediately after infusion, (*R,S*)-MCPG did not cause a chronic fall in baseline values. (B) For every animal, the percentage EPSP slope value obtained at each time point in the tetanized pathway was normalized to the value recorded in the control pathway (i.e. tetanized/control $\times$ 100). This method of analysis smoothes out fluctuations in the raw data. No differences in normalized LTP were found 50–60 min post-tetanus [200 mM (*R,S*)-MCPG = 116.6 $\pm$ 1.3%; vehicle = 112.7 $\pm$ 2.8%] or 110–120 min post-tetanus [200 mM (*R,S*)-MCPG = 109.8 $\pm$ 3.6%; vehicle = 110.3 $\pm$ 1.8%]. (C) 200 mM (*R,S*)-MCPG failed to block population spike LTP. Absolute increases in spike amplitude relative to baseline values are plotted for both tetanized and control pathways. Equivalent potentiation was seen in the tetanized pathway for each group 50–60 min post-tetanus [200 mM (*R,S*)-MCPG = 3.02 $\pm$ 0.69 mV; vehicle = 2.78 $\pm$ 0.49 mV] and 110–120 min post-tetanus [200 mM (*R,S*)-MCPG = 2.43 $\pm$ 0.89 mV; vehicle = 1.99 $\pm$ 0.30 mV]. Untetanized pathways likewise did not differ 50–60 min post-tetanus [200 mM (*R,S*)-MCPG = 0.08 $\pm$ 0.10 mV; vehicle = 0.13 $\pm$ 0.16 mV] or 110–120 min post-tetanus [200 mM (*R,S*)-MCPG = 0.43 $\pm$ 0.19 mV; vehicle = 0.32 $\pm$ 0.15 mV. (D) For each animal, the spike increase in the tetanized pathway was subtracted from that in the control pathway at each individual time point. No group differences in the normalized data were found 50–60 min post-tetanus [200 mM (*R,S*)-MCPG = 2.94 $\pm$ 0.71 mV; vehicle = 2.65 $\pm$ 0.46 mV] or 110–120 min post-tetanus [200 mM (*R,S*)-MCPG = 2.0 $\pm$ 0.94 mV; vehicle = 1.67 $\pm$ 0.35 mV].

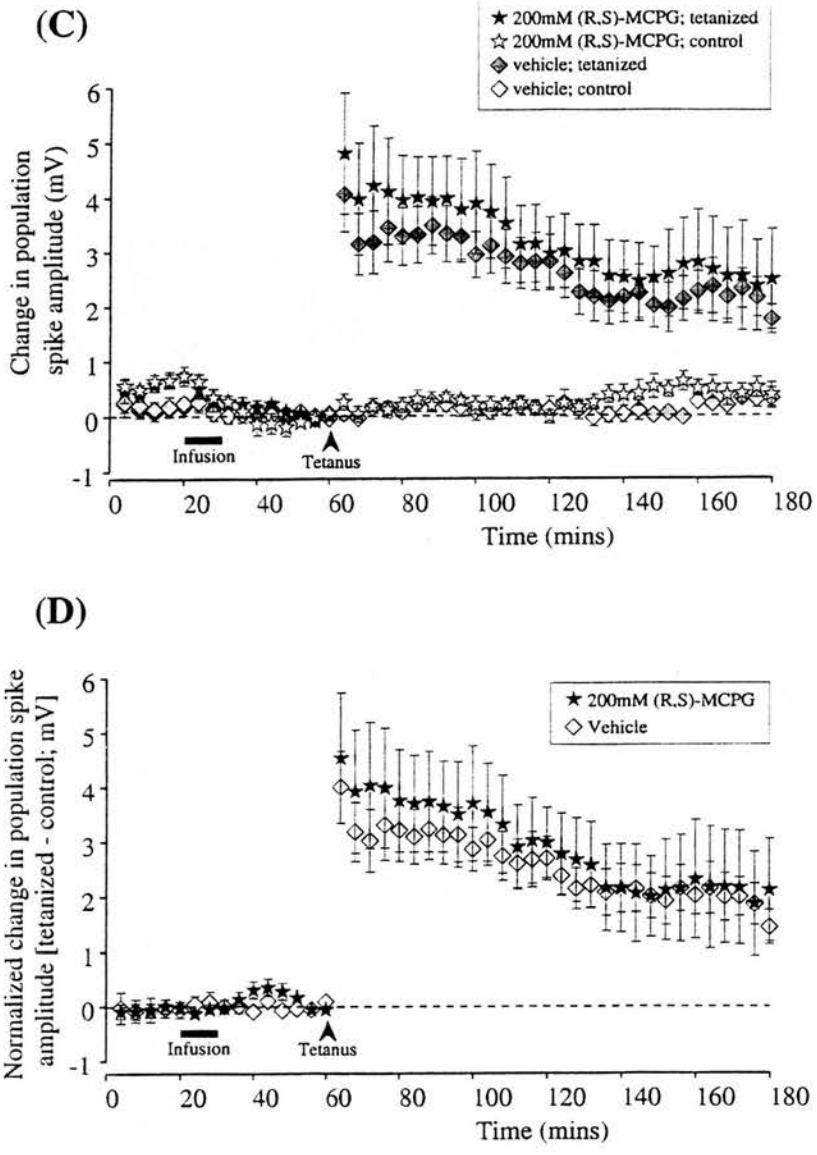

Fig. 2. Continued

normalized slope baseline [(tetanized/control) × 100] changed by more than 10% during the hour before tetanization, the results were not included in the final analysis.

EPSP slope LTP. MCPG infusion again failed to block LTP. Figure 2(A) shows the data for both control and tetanized pathways, normalized to the mean value 10 min prior to tetanization. Separate ANOVAs at different time points, in which tetanized and control pathway values were entered as sub-groups within each drug treatment group, revealed no significant drug group by pathway interaction 50–60 min post-tetanus [$F < 1$], or 110–120 min [$F < 1$]. The tetanized pathways were still significantly potentiated overall relative to the control pathways 110–120 min post-tetanus [$F(1,20) = 14.2$; $p < 0.01$]. *Post hoc* pairwise comparisons at this time point (Newman–Keuls) revealed that tetanized pathways were significantly potentiated relative to control pathways in each group (200 mM MCPG: $p < 0.05$; vehicle: $p < 0.05$); pairwise comparisons between the two tetanized pathways revealed no significant differences in the level of LTP; no differences were found between the control pathway values. A similar analysis of the level of PTP (0–4 min post-tetanus) also failed to reveal a group by pathway interaction [$F < 1$].

An ANOVA of absolute mean EPSP slope values over the 10 min before drug infusion, in which tetanized and control pathways were entered as sub-groups revealed no significant differences in initial slope values between drug groups [$F(1,20) = 3.64$; $p > 0.05$] or between tetanized and control pathways [$F(1,20) = 3.02$; $p > 0.05$]. A similar analysis of test-pulse stimulation intensities revealed no significant differences between groups [$F < 1$], or between pathways [$F(1,20) = 1.30$; $p > 0.2$].

In Fig. 2(B) the EPSP slope data are plotted using the

non-tetanized pathway as a within-subject control at each time point throughout the experiment, i.e. normalized value = [(tetanized/control) × 100]. Equivalent LTP was seen in both groups: no significant group differences were found 50–60 min post-tetanus [$F(1,10) = 1.22$; $p > 0.2$], or 110–120 min post-tetanus [$F < 1$].

The slight baseline changes noted in experiment 1 were observed in all animals infused with MCPG. However, no significant differences in EPSP slope were found between vehicle and MCPG control pathways 50–60 min post-tetanus [$F < 1$], indicating that these changes were transient and could not affect the level of LTP obtained. Comparison of the baseline period in Fig. 2(A) and (B) demonstrates that normalization of tetanized to control values smoothes out these baseline fluctuations, since both pathways are affected in the same way by MCPG infusion.

Population spike LTP. Figure 2(C) shows the population spike increase for both control and tetanized pathways, relative to the mean baseline value over the 10 min prior to tetanization: MCPG had no effect on the induction of spike LTP. An ANOVA in which tetanized and control pathways were entered as sub-groups revealed no group by pathway interaction in the level of LTP: 50–60 min post-tetanus [$F < 1$], 110–120 min post tetanus [$F < 1$] or PTP: 0–4 min post-tetanus [$F < 1$].

An ANOVA of mean absolute population spike amplitudes over the 10 min prior to drug infusion, in which tetanized and control pathways were entered as within-subject factors, revealed no significant differences between drug groups [$F < 1$], or between control and tetanized pathways [$F < 1$].

At each time point, the population spike change in a control pathway was subtracted from the corresponding change in the tetanized pathway; the normalized data are plotted in Fig. 2(D). MCPG and vehicle groups did not differ either 50–60 min post-tetanus [$F < 1$], or 110–120 min post-tetanus [$F < 1$].

Brain temperature. Infusion of MCPG caused a slight transient increase in brain temperature relative to vehicle-infused controls, similar to that described in experiment 1, but the mean temperature increase over the 30 min after drug infusion did not differ significantly between groups on this occasion [200 mM MCPG = 0.36 ± 0.15°C; vehicle = 0.12 ± 0.08°C; $F(1,10) = 2.51$; $p > 0.1$].

Experiment 3: does MCPG antagonize the electrophysiological effects of the mGLuR agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) in vivo?

The recording set-up was identical to that used in experiment 1. A thermistor was placed in the dentate gyrus contralateral to the stimulation and recording electrodes in order to record brain temperature. Spontaneous hippocampal activity was monitored by polygraph. Following a 20-min baseline period, rats were infused with 10 μl of either phosphate buffered saline ($n = 8$) or 200 mM (*R,S*)-MCPG ($n = 8$) over 10 min. Thirty minutes later, a 10 μl infusion of 1 mM (1*S*,3*R*)-ACPD was given, again over 10 min. A second infusion of (1*S*,3*R*)-ACPD was given 2 hr after the start of the first infusion of this drug in order to investigate the "wash-out" of (*R,S*)-MCPG. Recording was continued for a further 50 min after the end of this infusion. At the end of the experiment, six rats from each group were passively warmed, and regression lines were calculated relating EPSP slope to brain temperature. The experiment was later repeated with infusions of 4 mM (1*S*,3*R*)-ACPD following an initial infusion of 200 mM (*R,S*)-MCPG ($n = 4$) or PBS ($n = 4$). Brain temperature was not monitored in these rats.

Changes in spontaneous activity of the dentate gyrus. Figure 3(A) shows a representative polygraph trace recorded during and after the first 1 mM ACPD infusion in a rat previously infused with buffered saline: the rise in spontaneous activity was characteristic of ACPD infusion. No changes in spontaneous activity were seen during passive warming of animals. An equivalent polygraph trace recorded from a rat previously infused with 200 mM MCPG is shown in Fig. 3(B). On this occasion, the increase in noise was completely blocked.

Figure 3(C) shows the mean increase in amplitude of spontaneous activity in each group resulting from 1 mM ACPD infusion, sampled at 2-min intervals. Values were normalized to the mean amplitude 58–60 min after the start of the experiment. Spontaneous activity was not routinely recorded during infusion of PBS or 200 mM MCPG, since previous pilot studies had already established that no changes in noise levels resulted from infusion of these drugs (PBS: $n = 3$; 200 mM MCPG: $n = 5$; data not shown). The maximum increase in spontaneous activity from baseline values was 0.18 ± 0.29 mV in the MCPG pre-treated group, compared to 0.96 ± 0.22 mV in the PBS pre-treated group. An ANOVA of all data points from 60 to 80 min after the start of the experiment, in which time was entered as a within-subject factor, revealed a significant group difference [$F(1,14) = 7.39$; $p < 0.05$], and a significant group by time point interaction [$F(10,140) = 2.37$; $p < 0.05$]. Subsequent analysis of simple effects revealed that spontaneous activity levels were significantly higher in the PBS/ACPD group than in the MCPG/ACPD group for a period 6 min after the start of ACPD infusion until 2 min after the end of infusion (see graph legend). However, inspection of Fig. 3(C) suggests that prior infusion of MCPG unmasked a slight initial depression in spontaneous activity induced by ACPD.

The second infusion of 1 mM ACPD resulted in a slightly greater, but still transient, increase in spontaneous activity which was only partially blocked by prior infusion of MCPG 180 min earlier, suggesting incomplete wash-out of the drug (data not shown).

The increase in spontaneous activity resulting from the first infusion of 4 mM ACPD is shown in Fig. 3(D). This increase in noise was again blocked by MCPG: the

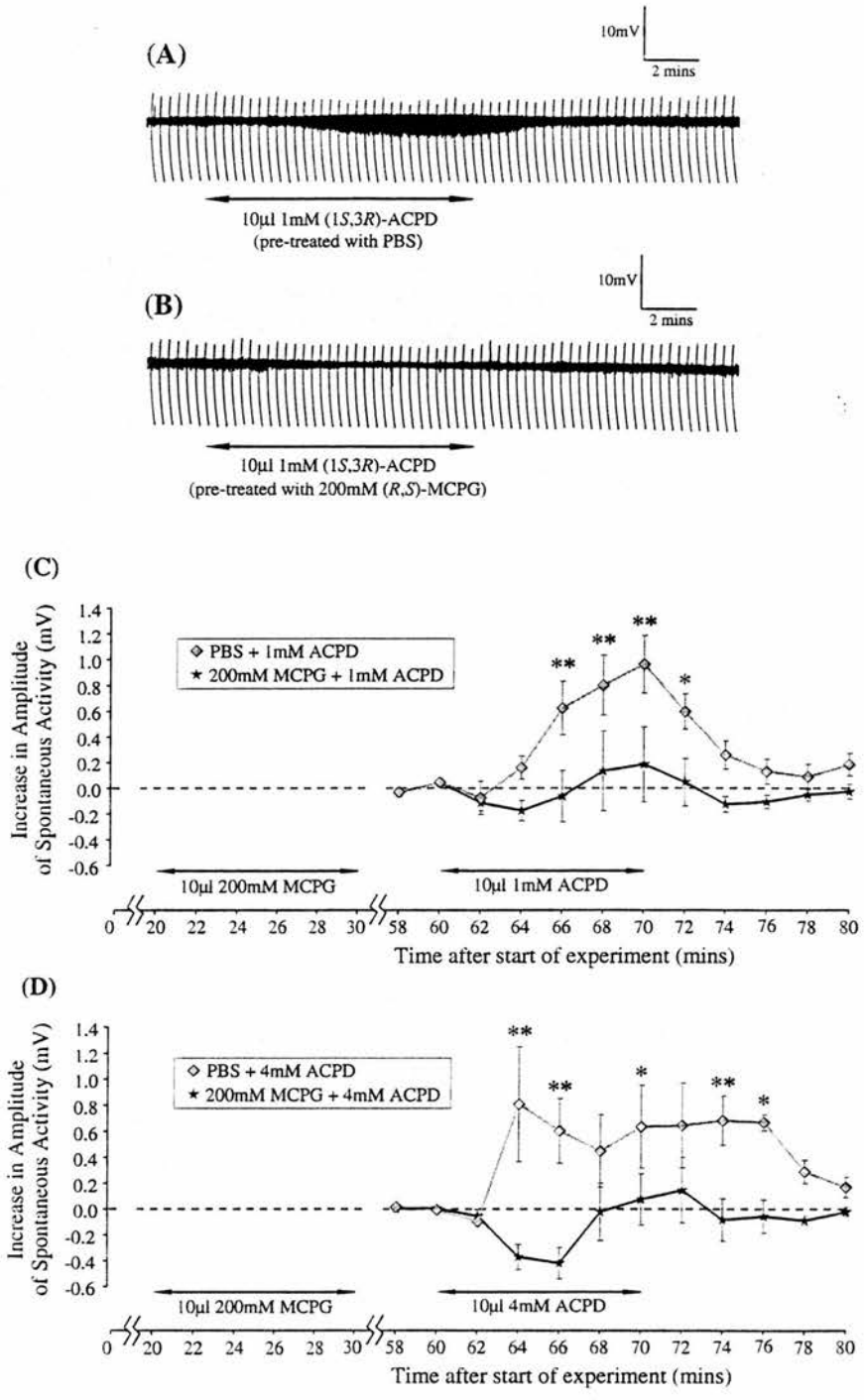

Fig. 3. (A) Polygraph chart record during a 10 μl 1 mM (1*S*,3*R*)-ACPD infusion, 30 min after infusion of 10 μl phosphate buffered saline. The observed increase in spontaneous activity was characteristic of (1*S*,3*R*)-ACPD infusion. (B) Polygraph chart record during a 10 μl 1 mM (1*S*,3*R*)-ACPD infusion, 30 min after infusion of 10 μl 200 mM (*R*,*S*)-MCPG. The rise in spontaneous activity was completely blocked. (C) Mean increase in amplitude (peak to trough) of spontaneous activity resulting from 10 μl 1 mM (1*S*,3*R*)-ACPD infusion measured directly from polygraph chart records at 2-min intervals. Values are normalized to the mean amplitude 58–60 min after the start of the experiment. Prior application of 10 μl 200 mM (*R*,*S*)-MCPG ($n = 8$) significantly reduced the increase in spontaneous activity induced by infusion of 1 mM (1*S*,3*R*)-ACPD 30 min later, relative to vehicle pre-treated controls ($n = 8$) ($^*p < 0.05$; $^{**}p < 0.01$). No changes in spontaneous activity levels were seen during either 200 mM (*R*,*S*)-MCPG or vehicle infusion (data not shown; see text). (D) Prior application of 10 μl 200 mM (*R*,*S*)-MCPG ($n = 4$) significantly reduced the increase in spontaneous activity induced by infusion of 10 μl 4 mM (1*S*,3*R*)-ACPD 30 min later, relative to vehicle-infused controls ($n = 4$) ($^*p < 0.05$; $^{**}p < 0.01$).

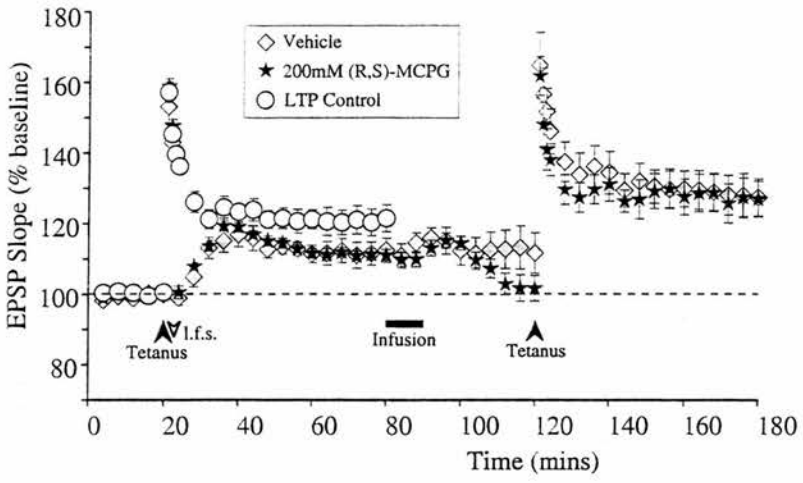

Fig. 4. Low frequency stimulation (5 Hz for 1 min) given 2 min after a high frequency tetanus resulted in significant EPSP slope depotentiation relative to controls given only the tetanus [EPSP slope 50–60 min post-tetanus: control = 120.2 ± 3.4% ($n = 8$); depotentiated (vehicle and 200 mM (*R,S*)-MCPG groups combined) = 111.5 ± 1.9% ($n = 15$)]. Subsequent infusion of 10 μl 200 mM (*R,S*)-MCPG ($n = 7$) or vehicle ($n = 8$) resulted in equivalent LTP after a second tetanus.

maximum increase in the MCPG pre-treated group was 0.14 ± 0.25 mV, compared to 0.81 ± 0.44 mV in the PBS pre-treated group. Analysis of all time points from 60 to 80 min after the start of the experiment revealed a significant overall group difference between MCPG and PBS treated rats [$F(1,6) = 12.01$; $p < 0.05$], together with a significant group by time point interaction [$F(10,60) = 2.58$; $p < 0.05$]. Significant group differences at individual time points are indicated in Fig. 4(D). The slight depression in spontaneous activity unmasked by ACPD infusion in the presence of MCPG appears more marked after 4 mM ACPD infusion than after 1 mM ACPD. Furthermore, the response to 4 mM ACPD in the presence of PBS only appears to be biphasic, perhaps reflecting the superposition of a smaller depression of activity upon the larger increase in noise (see Discussion).

The second infusion of 4 mM ACPD resulted in a slightly greater increase in spontaneous activity which again was only partially blocked by by prior infusion of MCPG 180 min earlier (data not shown). However, two of the four rats initially infused with PBS showed evidence of epileptiform activity during the second ACPD infusion. Such activity was never observed in rats initially infused with MCPG.

Changes in EPSP slope. Infusion of ACPD was associated with a transient dose-dependent rise in EPSP slope; an effect not blocked by MCPG (data not shown). However, in those rats implanted with thermistors, the rise in EPSP slope was typically accompanied by a small rise in brain temperature whose time course paralleled that of the increase in slope. Calculation of the expected rise in slope based on the observed increase in temperature in these rats revealed that the observed slope increase was no greater than predicted by the change in temperature alone.

Experiment 4: does prior depotentiation reveal an MCPG-sensitive component of LTP in vivo?

The recording set-up was identical to that used in experiment 1. A 20-min baseline period was recorded, followed by a high frequency tetanus. However, 2 min after tetanization, a 1-min period of 5-Hz stimulation was applied to most rats. One hour after the first tetanus, these animals were infused with either 200 mM (*R,S*)-MCPG ($n = 7$) or vehicle ($n = 8$). Half an hour after the end of drug infusion, a second tetanus was given, and LTP was followed for 1 hr. A control group ($n = 8$) also received the first tetanus, but no depotentiating stimulation. LTP was followed for 1 hr in these animals, and their experiment ended at the point when drug infusion would have begun for the other groups.

Tetanus 1 and depotentiation. Significant depotentiation was induced by 5-Hz stimulation given 2 min after tetanization. An ANOVA of the amount of potentiation 50–60 min after the first tetanus, in which all depotentiated animals were compared with controls, revealed a significant reduction in EPSP slope potentiation [$F(1,21) = 5.87$; $p < 0.05$; Fig. 4]. However, the depotentiation was incomplete; only two of the 15 rats receiving 5-Hz stimulation showed less than 5% LTP after 1 hr; all control rats showed more than 5% LTP. No significant group differences were found in the amount of PTP 0–2 min post-tetanus [$F < 1$]. An analysis of population spike depotentiation gave similar results (data not shown).

No significant group differences were found in baseline stimulation intensity [$F < 1$]; initial spike amplitude over the 10 min prior to tetanus 1 [$F(2,20) = 1.19$; $p > 0.3$]; and absolute slope magnitude prior to tetanus 1 [$F(2,20) = 2.02$; $p > 0.1$].

Drug infusion and tetanus 2. Infusion of MCPG caused

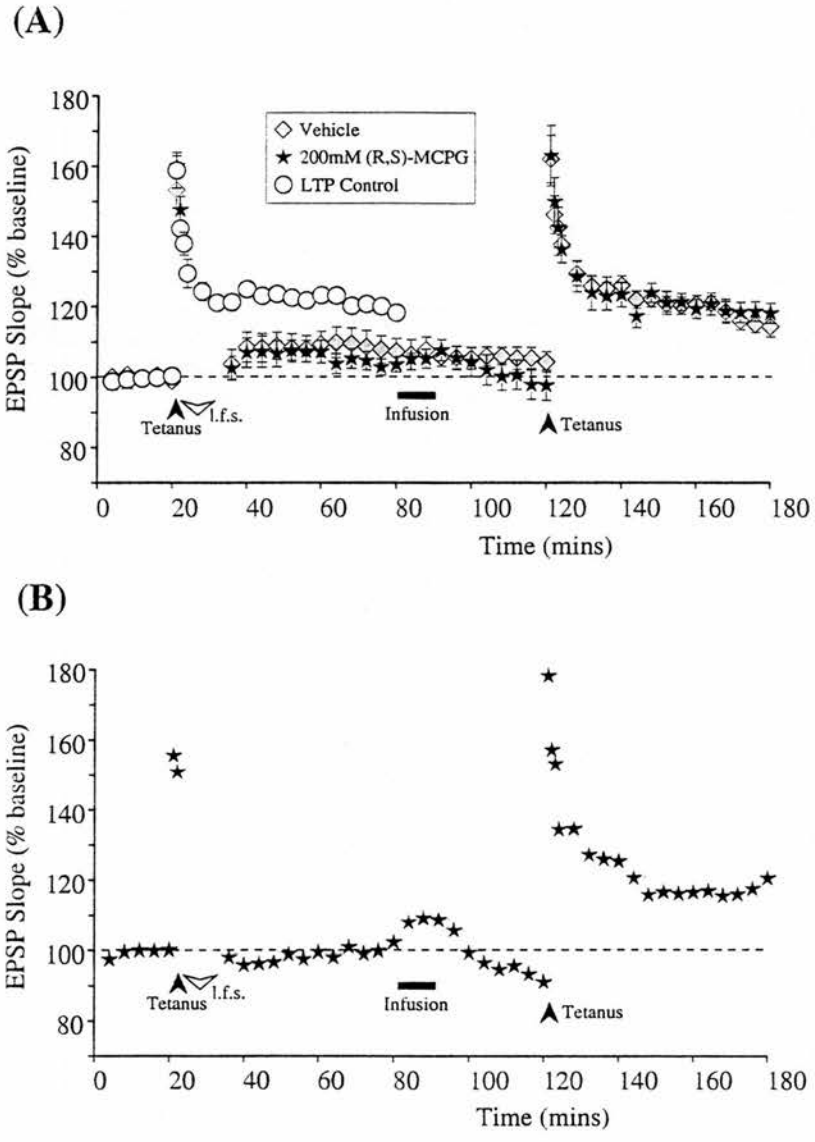

Fig. 5. (A) Low frequency stimulation (5 Hz for 10 min) given 2 min after a high frequency tetanus results in significant EPSP slope depotentiation relative to controls given only the tetanus [EPSP slope 50–60 min post-tetanus: control = 119.3 ± 1.4% ($n = 6$); depotentiated (200 mM (*R,S*)-MCPG and vehicle groups combined) = 105.6 ± 2.5% ($n = 12$)]. Subsequent infusion of 10 μl 200 mM (*R,S*)-MCPG ($n = 6$) or vehicle ($n = 6$) resulted in equivalent LTP after a second tetanus. (B) Infusion of 10 μl 200 mM (*R,S*)-MCPG did not block EPSP slope potentiation, even in individual animals showing complete depotentiation, such as the example given here.

a marked fall in baseline slope values (see Discussion), but failed to block LTP after the second tetanus. The amount of potentiation 50–60 min after the second tetanus, normalized to the original baseline, did not differ between MCPG and vehicle groups [slope LTP: $F < 1$; Fig. 4].

However, since depotentiation was not completely effective, it might be more appropriate to re-normalize the data to the slightly elevated baseline prior to the second tetanus. The 10 min prior to drug infusion were chosen, rather than the 10 min immediately before tetanization, in order that MCPG-induced baseline changes would not confound the results. Even when analysed in this way, no significant differences in LTP 50–60 min after tetanus 2 were found between the two groups [$F < 1$; data not shown].

Experiment 5: does a more effective depotentiation protocol reveal an MCPG-sensitive component of LTP?

All procedures were identical to those described in experiment 3, except that 5-Hz stimulation was continued for 10 min instead of 1 min.

Tetanus 1 and depotentiation. An ANOVA of the amount of EPSP slope LTP 50–60 min after tetanus 1, in which all depotentiated animals ($n = 12$) were compared with controls ($n = 6$), revealed a highly significant reduction in the depotentiated group [$F(1,16) = 13.3$; $p < 0.01$; Fig. 5(A)]. No significant differences were

found in the amount of PTP 0–2 min post-tetanus [$F < 1$]. An analysis of population spike depotentiation gave similar results (data not shown).

No group differences were found in baseline stimulation intensity [$F < 1$]; initial spike amplitude over the 10 min before tetanus 1 [$F < 1$]; and absolute slope magnitude before tetanus 1 [$F < 1$].

This protocol was slightly more effective than that used previously. A comparison of the amount of EPSP slope LTP following 5-Hz stimulation for 1 min (experiment 4) and the amount following 10 min (experiment 5) revealed a trend towards greater depotentiation in the latter [experiment 4 = 111.46 ± 1.91%; experiment 5 = 105.55 ± 2.25%; $F(1,25) = 3.59$; $p < 0.1$]. Furthermore, EPSP slope values returned to within 5% of baseline or less in seven of the 12 rats given 10 min of 5 Hz, compared to only two out of 15 given 1 min of low frequency stimulation. No significant differences in non-depotentiated control LTP were seen between experiments 4 and 5 [$F < 1$]; all controls showed more than 5% LTP.

Drug infusion and tetanus 2. MCPG did not block LTP following the second tetanus. The amount of potentiation 50–60 min after the second tetanus, normalized to the original baseline, did not differ between MCPG and vehicle groups [$F < 1$; Fig. 5(A)]. This was true even in individual cases where complete depotentiation was achieved (Fig. 5(B)). The same result was obtained when the results were re-normalized to the pre-infusion baseline (data not shown). These results confirm that the inability of MCPG to block LTP is unaffected by prior depotentiation.

DISCUSSION

The experiments described in this study have consistently revealed that intraventricular infusion of a high dose of (*R,S*)-MCPG in urethane-anaesthetized rats does not result in a block of LTP in the dentate gyrus. This result holds whether or not LTP is assessed relative to a pre-tetanus baseline, to a concurrently stimulated control pathway, or is induced in a pathway previously subject to depotentiation.

In experiment 1, i.c.v. infusion of either 20 mM or 200 mM (*R,S*)-MCPG failed to cause a significant reduction in the level of LTP up to 2 hr after tetanization. It is unlikely that this was due to any failure of the drug infusion protocol as 30 mM D-AP5 administered by the same route caused a total block of LTP. Surprisingly, the infusion of AP5 resulted in a slight increase in the EPSP slope baseline. AP5 is known to reduce population spike amplitude in the dentate gyrus (Errington *et al.*, 1987), an effect noted in the present study (data not shown). Nevertheless, increases in EPSP slope have not been reported previously. Although the reason for the increase in EPSP slope associated with AP5 infusion is unknown, data from two rats in which recordings were made bilaterally reveal that the slope increase in untetanized pathways persisted for the duration of the experiment and rules out the possibility that a residual potentiation was being masked.

Inspection of Fig. 1(A) reveals that slightly less LTP was observed in the 200 mM MCPG group compared to controls, though this effect was not found to be statistically significant. However, prior to tetanization, the baseline was falling in this group, an effect which, though slight, was fairly consistent across animals and may reflect a small metabotropic contribution to normal synaptic transmission. It was recently found that MCPG infusion into the basolateral amygdala causes a transient depression of baseline responses (Keele *et al.*, 1995). The authors attribute this to the action of MCPG as an agonist at presynaptic mGluR "autoreceptors", but such effects have not previously been described in the hippocampus. Nevertheless, a chronic baseline fall might give rise to an apparent block of LTP under some circumstances. In order to control for this possibility experiment 2 was conducted using bilateral stimulation and recording, with the one pathway serving as an untetanized control. The results confirm the finding of experiment 1, that MCPG is ineffective in blocking LTP. Furthermore, the slight baseline changes caused by MCPG are short-lasting and could not contribute to the level of potentiation 2 hr after tetanization. It appears, however, that the baseline fall caused by MCPG is more marked after the induction of LTP followed by incomplete depotentiation (Fig. 4) than the fall occurring after more effective depotentiation (Fig. 5(A)), or when MCPG is applied to an untetanized pathway (Fig. 1(A), Fig. 2(A)]. It is conceivable that tetanization followed by 5-Hz stimulation results in a residual mGluR-dependent potentiation which returns to baseline after MCPG application, although MCPG has been shown to have no effect on LTP when applied after a high frequency tetanus alone (Riedel *et al.*, 1995). Nevertheless, inspection of the data from individual rats suggests that the size of the fall in EPSP slope following MCPG infusion in experiments 4 and 5 is independent of the amount of LTP remaining after tetanization and 5-Hz stimulation (data not shown). This finding is inconsistent with the suggestion that the residual LTP after depotentiation is mGluR-dependent. However, the reason for the difference in baseline changes between experiments is not known.

It is unlikely that changes in brain temperature had a significant effect on the relative level of LTP recorded in experiments 1 and 2. In Fig. 1(D), the slight increase in brain temperature of approximately 0.5°C associated with MCPG infusion appeared to be transient. No significant temperature differences between groups were found 2 hr after tetanization, although the mean brain temperature of MCPG-treated rats was about 0.4°C higher at this point than in vehicle-infused controls. Nevertheless, it is unlikely that a block of LTP with 200 mM MCPG was masked by a small temperature-dependent increase in EPSP slope in this group since in experiment 2, vehicle-infused controls showed brain

temperatures slightly (but again non-significantly) higher than MCPG-infused animals 2 hr after tetanization (data not shown), yet a block of LTP was still not obtained with MCPG.

It could be argued that the above results simply reflect an insufficient blockade of mGluR activation in the dentate gyrus. For instance, intraventricular infusion of a drug results in an unknown distribution and concentration within the target structure; these are problems associated with most *in vivo* studies. Nevertheless, in experiment 3, MCPG infusion was found to inhibit the rise in spontaneous activity induced by infusion of the mGluR agonist, (1*S*,3*R*)-ACPD. Previous studies have established that application of ACPD has a number of direct excitatory effects on CA1 pyramidal cells, including inhibition of the slow afterhyperpolarization current, and blockade of spike frequency adaptation (Desai and Conn, 1991; Davies *et al.*, 1995). A similar increase in the excitability of dentate granule cells may underlie the rise in spontaneous activity recorded in the present study. The fact that MCPG is effective in blocking an electrophysiological response to ACPD confirms that the drug reaches ACPD-activated mGlu receptors. The blockade was equally effective at both 1 mM and 4 mM doses of ACPD. Note that the application of 4 mM (1*S*,3*R*)-ACPD following 200 mM (*R*,*S*)-MCPG reflects the agonist: antagonist ratio typically used *in vitro* (e.g. 10 μM (1*S*,3*R*)-ACPD: 500 μM (*R*,*S*)-MCPG; see Bashir *et al.*, 1993). However, it is possible that the reduction in ACPD-induced spontaneous activity may not reflect the antagonism of mGluRs within the dentate gyrus, but that MCPG may be blocking a network-mediated action of ACPD originating elsewhere in the brain. It has also been reported that MCPG exhibits greater potency in antagonizing ACPD-mediated responses, compared to those of glutamate (Brabet *et al.*, 1995; Littman and Robinson, 1994). Hence, it might still be argued that glutamate release during tetanic stimulation could be sufficient to overcome the competitive antagonism of MCPG. However, slice studies indicate that MCPG can block the LTP induced by 100-Hz stimulation for 1 sec (i.e. 100 pulses; see Bortolotto *et al.*, 1995), a protocol likely to result in greater glutamate release than the 20 pulses per train used in the present study.

Although MCPG successfully antagonized the increase in spontaneous activity induced by ACPD, it unmasked a dose-dependent depression (see Fig. 3(C) and (D)). This may reflect a preferential antagonism of the excitatory actions of class I mGluRs, revealing an inhibitory effect of ACPD, perhaps mediated by class II mGluRs not blocked by MCPG. The evidence in support of this is that MCPG competitively inhibits ACPD-induced phosphoinositide hydrolysis, but does not inhibit the agonist-induced depression of forskolin-stimulated cAMP formation in adult rat hippocampal slices (Riedel *et al.*, 1995). The qualification of this argument is that MCPG has been found to inhibit the latter effect in cortical brain slices and in cells expressing mGluR2 (Kemp *et al.*, 1994; Hayashi *et al.*, 1994). However, the results of Riedel *et al.* (1995) suggest that it is the activation of class I mGluRs which is necessary for the induction of LTP, so the failure of MCPG to block a possible class II mGluR-mediated effect of ACPD in the present study is unlikely to account for the failure of MCPG to block LTP.

Infusion of ACPD caused only a transient increase in EPSP slope associated with a slight increase in brain temperature. Slow-onset potentiation of the kind reported by Manahan-Vaughan and Reymann (1995) using 4 mM (1*S*,3*R*)-ACPD in the dentate gyrus *in vivo* was not seen. However, the results of the present study are consistent with those of Davis and Laroche (1996), who reported a rapid-onset but transient rise in EPSP slope in the dentate after infusion of 10 mM (1*S*,3*R*)-ACPD.

It has been suggested that mGluR activation may regulate plasticity by turning on a "molecular switch" which eliminates the requirement for further mGluR activation in the induction of LTP; this switch can be reset by depotentiating stimulation (Bortolotto *et al.*, 1994). However, in experiment 4, MCPG remained ineffective in blocking LTP even after significant depotentiation. Nevertheless, 1 min of low frequency stimulation did not completely erase LTP (potentiation was reduced to within 5% of baseline in only two of 15 animals tested); it could be argued that a more effective depotentiation protocol might produce a different result. However, the results of experiment 5 confirm that the inability of MCPG to block LTP is unaffected by prior depotentiation. In this case, 10 min of 5-Hz stimulation resulted in LTP of less than 5% in seven out of 12 animals tested, but MCPG again failed to block LTP, even in those individual animals showing complete depotentiation prior to drug infusion and re-tetanization. It is conceivable that LTP reaches a ceiling after the second tetanus, thus masking differences in potentiation between the groups; LTP is of equal magnitude in the MCPG group whose baseline is low prior to tetanus 2 and the aCSF group whose pre-tetanus baseline is higher. However, inspection of Fig. 2(A) suggests that these baseline differences are likely to be transient. Moreover, it is unlikely that LTP is saturated in Fig. 5(A), since the EPSP slope 1 hr after tetanus 2 is only about 20% above the baseline level prior to tetanus 1.

The above results are consistent with those of Selig *et al.* (1995) and Thomas and O'Dell (1995) who failed to replicate the molecular switch findings of Bortolotto and Collingridge (1994) in area CA1 *in vitro*. Whilst the results of the present study do not rule out the possibility that the sensitivity of LTP to MCPG may, under certain circumstances, be governed by the position of a molecular switch, it is unlikely that such a possibility can account for the negative results described here.

Nevertheless, it is interesting that a period of 5-Hz stimulation given 2 min after tetanization results in significant depotentiation: 10 min of this depotentiating stimulation resulted in a mean of only 5.6 $\pm$ 2.5% LTP

after 1 hr, compared to 19.3 ± 1.4% in tetanized-only controls. Depotentiation has been reliably obtained in area CA1 *in vitro* in a number of different laboratories (Fujii *et al.*, 1991; Larson *et al.*, 1993; Bashir and Collingridge, 1994; O'Dell and Kandel, 1994); it is notable that low frequency stimulation in the theta range, such as the 5 Hz used in the present study, has been found to be optimal for reversing LTP. Initial reports suggested that depotentiation could be obtained in area CA1 *in vivo*, both in anaesthetized (Barrionuevo *et al.*, 1980) and freely-moving rats (Stäubli and Lynch, 1990), but others have failed to induce depotentiation in either the CA1 region or dentate gyrus under similar circumstances (Errington *et al.*, 1995). Recent work has demonstrated that, at least in area CA1 *in vitro*, the percentage reversal of LTP declines steeply as the interval between tetanus and low frequency stimulation is increased: depotentiation is most effective when 5-Hz stimulation is given in the first few minutes following tetanization (Stäubli and Chun, 1996). As the authors note, this timescale matches the consolidation period (1–3 min) during which LTP is susceptible to disruption by brief periods of hypoxia and application of adenosine ~~antagonists~~ (Arai *et al.*, 1990a,b); it may be significant that low frequency stimulation was given within this time-window in the present study. The existence of a critical period for the induction of depotentiation may explain the negative results obtained by Errington *et al.*, as they allowed 30 min to intervene between tetanization and low frequency stimulation. Further studies will be necessary to determine whether depotentiation in the dentate gyrus *in vivo* exhibits the same time-dependent properties as those found in CA1 *in vitro*.

The failure of MCPG to block LTP in the dentate gyrus of urethane-anaesthetized rats is consistent with the results of one recent study conducted under similar conditions (Bordi and Ugolini, 1995); an earlier report described a reduction in the percentage EPSP slope potentiation after acute intraventricular infusion, but no block of population spike potentiation (Richter-Levin *et al.*, 1994). A complete block of LTP was seen only during direct perfusion of MCPG into the dentate gyrus via a push–pull cannula. However, it has been reported that urethane antagonizes the excitation induced by application of excitatory amino acid agonists, including NMDA, in isolated spinal cord (Evans and Smith, 1982). In another study, urethane anaesthesia was found to depress dentate gyrus granule cell excitability and the strength of synaptic responses (Shirasaka and Wasterlain, 1995). The recent finding that *Thy-1* knockout mice show a complete block of dentate LTP under urethane anaesthesia, but express significant LTP in the freely-moving state, demonstrates that the effects of anaesthesia cannot be ignored (Nosten-Bertrand *et al.*, 1996; Errington *et al.*, 1997). Consistent with this result, it is found that stronger tetanization parameters are required to induce LTP in area CA1 of urethane-anaesthetized rats, compared to the freely-moving state (Riedel *et al.*, 1994). It has been reported that MCPG does not block the LTP induced in area CA1 *in vitro* by strong tetanization protocols, such as theta burst stimulation (Brown *et al.*, 1994). The weaker tetanization parameters necessary in freely-moving rats may unmask a modulatory role of mGluRs, accounting for reports of a complete block of LTP by MCPG under these circumstances (see Riedel *et al.*, 1996). It is worth noting, however, that the tetanus parameters chosen in the current study were in fact quite modest, typically yielding just over 15% LTP after 2 hr (see experiment 1). Nevertheless, it will be necessary to extend the experiments described here to freely-moving animals before any firm conclusions about the involvement of mGluRs in LTP *in vivo* can be drawn.

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Time-dependent reversal of dentate LTP by 5 Hz stimulation

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IELD potential recordings were made from the dentate yrus of urethane-anaesthetized rats in order to inves-gate the ability of 5 Hz stimulation to reverse long-rm potentiation (LTP) induced by a high frequency tanus. A 10 min train of 5 Hz was found to reverse TP in a time-dependent fashion: as the interval between tanus and 5 Hz was increased, LTP became progres-vely less susceptible to reversal. If 10 min or 30 min ıtervened between tetanization and 5 Hz stimulation, TP was unaffected. These results indicate that dentate TP *in vivo* exhibits a similar limited time window of ulnerability to reversal by low frequency stimulation) that previously reported in area CA1 *in vitro*. *leuroReport* 9: 3775–3781 © 1998 Lippincott Williams & Vilkins.

ey words: Depotentiation; Hippocampus; Long-term otentiation (LTP); Low frequency stimulation

ntroduction

After many years of research into the role of long-erm potentiation (LTP) in learning,[1–3] considerable ttention has recently been devoted to the phenom-non of depotentiation, the activity-dependent eversal of LTP. A number of laboratories have btained depotentiation in area CA1 *in vitro* using a ontinuous train of 1–5 Hz stimulation.[4–8] However, *n vivo* studies of the reversal of CA1 LTP have enerated mixed results, with some authors reporting uccessful depotentiation using low frequency stim-lation,[9–11] and others failing to obtain any effect.[12]

In the dentate gyrus, heterosynaptic depotentia-tion has been observed *in vivo*[13] and homosynaptic depotentiation has been successfully induced in slices taken from immature rats.[14] However, homosynaptic depotentiation is generally not induced by the delivery of low frequency stimulation to the medial perforant path–dentate gyrus projection of adult rats *in vivo*.[12,15] One factor that may be critical for the successful induction of depotentiation is the interval between tetanization and low frequency stimulation. A systematic investigation of this possibility in area CA1 *in vitro* revealed that the percentage reversal of LTP declines steeply as the interval between tetanus and low frequency stimulation is increased.[4] Depotentiation was found to be most effective when 5 Hz stimulation was delivered within minutes of tetanization.

I have previously reported that a 2 or 10 min period of 5 Hz stimulation results in a significant reversal of dentate LTP *in vivo* when delivered 2 min after a high frequency tetanus.[16] In the present study, I investigated the possibility that dentate LTP *in vivo*, like CA1 LTP *in vitro*, exhibits a limited time window of vulnerability to reversal by 5 Hz stimulation.

Materials and Methods

Adult male Lister-hooded rats (250–500 g) were given *ad lib* access to food and water and were maintained on a 12:12 h light:dark cycle. Experiments were conducted under urethane anaesthesia (1.5 g/kg, i.p.), with the rat mounted in a stereotaxic frame (Kopf, Tujunga, CA) with skull horizontal. The animal's temperature was monitored by a rectal probe and maintained at 36.2 ± 0.2°C using an isothermic heating blanket. PTFE-coated, platinum/iridium electrodes were lowered into the left hippocampal formation in order to record positive-going field potentials. A bipolar stimulating electrode was positioned in the angular bundle of the perforant path (AP = 7.5 mm; L = 4.0 mm) and a monopolar recording electrode in the hilus of the dentate gyrus (AP = 3.5 mm, L = 2.0 mm). Field EPSPs were amplified using a polygraph (Grass Instruments, Quincy, MA). The initial slope of the field EPSP (measured by linear regression between two fixed time points), and the population spike amplitude, were monitored on-line by an Acorn A5000 computer running specialized software.

Stimulation consisted of monophasic pulses, the intensity of which was adjusted to elicit a population spike of 2–4 mV. The pulse width was 50 μs, except during a high frequency tetanus or 5 Hz stimulation, when the pulse width was doubled. Test pulses were

delivered at 0.05 Hz throughout, except during the recording of input/output (I/O) curves, when this frequency was increased to 0.1 Hz.

Once stable EPSPs had been obtained, an I/O curve was recorded, consisting of four pulses at each of 10 stimulation intensities ranging from 0.1 to 1 mA. A baseline period of 20 min followed, after which a high frequency tetanus was delivered. Tetanic stimulation consisted of three trains of 50 pulses of 100 μs duration, delivered at 250 Hz, with 10 s between trains. A single test pulse of 100 μs duration was given 10 s after the final tetanus train to provide an early measure of post-tetanic potentiation (PTP). After a variable interval, measured from the final tetanus train, a 10 min period of 5 Hz stimulation was delivered. The intervals tested were as follows: 10 s ($n = 7$); 30 s ($n = 6$); 2 min ($n = 7$); 10 min ($n = 7$); 30 min ($n = 6$). Additional control groups received either 5 Hz only, with no tetanus ($n = 6$), or tetanic stimulation only, with no subsequent 5 Hz period ($n = 6$). At the end of the 5 Hz stimulation period, test pulse stimulation was resumed until 1 h had passed since tetanization. A second I/O curve was then recorded. The above protocol is summarized in Fig. 1.

A further group of rats received 10 min of 1 Hz, rather than 5 Hz, starting 10 s after tetanization ($n = 6$). Additional LTP controls were interleaved with this 1 Hz group ($n = 6$). All other aspects of the protocol were identical to that described above.

Separate groups of rats received intraventricular infusions of 200 mM (*R,S*)-α-methyl-4-carboxyphenylglycine (MCPG) prior to tetanization only ($n = 3$), or tetanization followed 10 s later by 10 min of 5 Hz stimulation ($n = 3$). In these animals, two stainless steel injection cannulae connected by plastic tubing to microsyringes, were lowered into the left and right lateral ventricles (AP = –0.9 mm; L = ± 1.3 mm). (*R,S*)-MCPG was dissolved in 1 M NaOH to form an equimolar solution, then diluted with phosphate buffered saline to achieve a concentration of 200 mM. A total volume of 10 μl (5 μl per ventricle) was infused over 10 min, starting 40 min prior to tetanization. Otherwise, the protocol was identical to that described above.

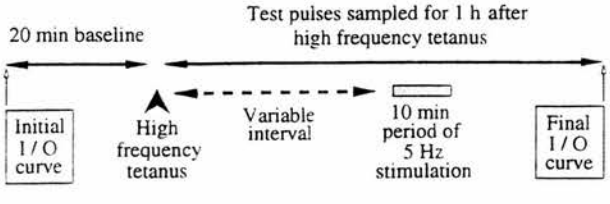


FIG. 1. Summary of experimental design. 5 Hz stimulation was delivered at intervals ranging from 10 s to 30 min following a high frequency tetanus. See text for details.

All statistical comparisons were carried out using analysis of variance (ANOVA) unless otherwise stated. Numerical values are given as mean ± s.e. throughout.

Results

Reversal of EPSP slope potentiation: Figure 2A shows the amount of EPSP slope potentiation at increasing delays between tetanus and 5 Hz stimulation, normalized to the mean value recorded over the 10 min prior to tetanization. For reference, the 5 Hz only and tetanus only control groups are included in each individual graph. High frequency tetanization alone reliably induced LTP; 5 Hz stimulation alone generally had little effect, although a slight increase in EPSP slope was sometimes observed. The degree of LTP reversal declined as the interval between tetanus and 5 Hz was increased; 5 Hz stimulation delivered 10 min or more after tetanization had little effect other than causing a transient depression of responses, typically lasting less than 10 min.

The mean percentage LTP in each group, measured over the final 10 min of the experiment (50–60 min after tetanization) is plotted in Fig. 2B. An ANOVA revealed a highly significant overall effect of group ($F(6,38) = 10.24$; $p < 0.0001$). A significant reversal of LTP was only seen when less than 10 min intervened between tetanus and 5 Hz (*post-hoc* Dunnett's pairwise comparisons between tetanus only and all other groups; see figure legend). Delivery of 5 Hz starting 10 s after tetanization resulted in a complete reversal of LTP. No significant difference was found between the amount of LTP in this group and that in the 5 Hz only group ($p > 0.9$; *post hoc* Dunnett's pairwise comparison). In fact, only the 10 min, 30 min and tetanus only groups exhibited significant LTP relative to the 5 Hz only condition ($p < 0.01$ in each case; Dunnett's).

No significant group differences were found in absolute mean EPSP slope values over the 10 min before tetanization ($F < 1$). A measure of initial slope PTP was calculated for all tetanized groups by dividing the value recorded 10 s after the final tetanus train by the value obtained from the first pulse of the first tetanus train, then multiplying by 100 (data not shown). No group differences were found in this measure of early slope PTP ($F < 1$), indicating that the initial potentiation induced by tetanization was identical across groups.

Reversal of population spike potentiation: The population spike data were generally consistent with the EPSP slope data. Figure 2C shows the absolute increase in population spike amplitude relative to the

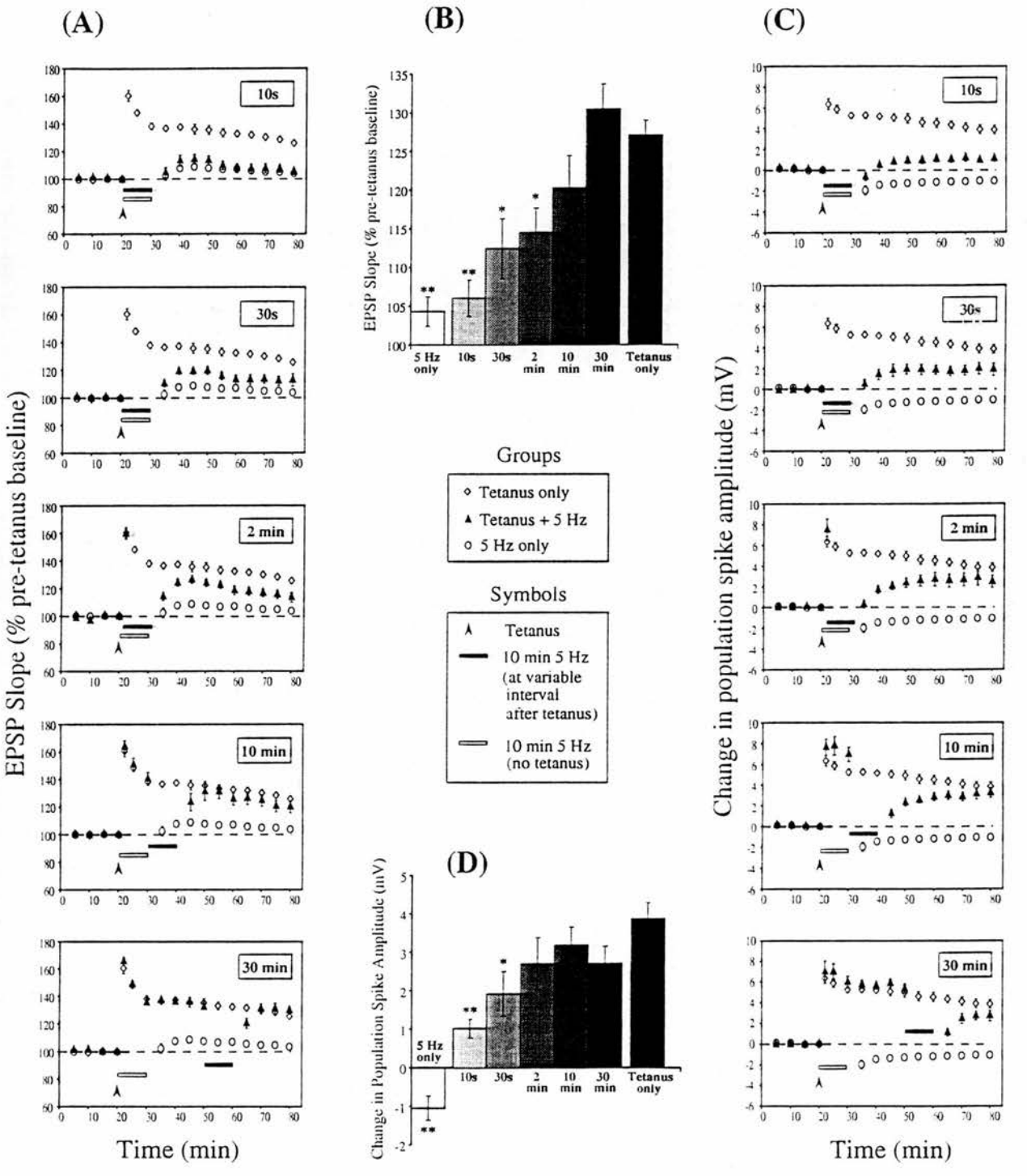


FIG. 2. (**A**) EPSP slope values normalized to the pre-tetanus baseline at increasing delays between tetanization and 5 Hz stimulation. For reference, tetanus only and 5 Hz only groups are included in each individual panel. (**B**) Mean EPSP slope values 50–60 min after tetanization. Asterisks indicate groups showing significantly less LTP than tetanus only controls (*$p < 0.05$; **$p < 0.01$). (**C**) Changes in population spike amplitude normalized to the pre-tetanus baseline. (**D**) Mean increase in population spike amplitude 50–60 min after tetanization. Asterisks indicate a significant reversal of LTP relative to tetanus only controls (*$p < 0.05$; **$p < 0.01$).

pre-tetanus baseline at all intervals between tetanus and 5 Hz. Tetanus only and 5 Hz only control groups are again included in each individual graph. High frequency tetanization alone induced a lasting increase in population spike amplitude; 5 Hz stimulation alone caused a small but lasting depression of the population spike. Consistent with the EPSP slope findings, the efficacy of population spike LTP reversal declined as the interval between tetanus and 5 Hz stimulation was increased. The

delivery of 5 Hz starting 2 min or more after tetanization caused only a transient depression in population spike amplitude, which although outlasting the EPSP slope depression, typically recovered within about 20 min.

The mean population spike increase 50–60 min after tetanization is shown in Fig. 2D. The overall effect of the group was highly significant ($F(6,38) = 11.64$; $p < 0.0001$). The 5 Hz only, 10 s and 30 s groups showed significantly less LTP than tetanus only controls (*post-hoc* Dunnett's pairwise comparisons; see figure legend); delivery of 5 Hz stimulation 2 min or more after tetanization had no significant effect.

No significant group differences were found in absolute baseline population spike amplitude over the 10 min prior to tetanization ($F < 1$); the test pulse stimulation intensities required to elicit population spikes of this amplitude did not differ across groups ($F < 1$). An analysis of population spike PTP recorded 10 s after tetanization revealed no group differences in initial potentiation ($F(5,33) = 1.41$; $p > 0.2$).

Changes in EPSP-spike (E-S) coupling after 5 Hz stimulation: Mean EPSP slope and population spike amplitude values were calculated for each stimulation intensity sampled during both the initial and final I/O curves. The maximum mean EPSP slope and population spike amplitude recorded during the initial I/O curve were arbitrarily assigned values of 100%, to which all other values were normalized. For each rat, scatter plots relating EPSP slope to population spike amplitude were constructed both before and after tetanization and/or 5 Hz stimulation.

Figure 3 shows mean E-S plots constructed from the initial and final I/O curves, i.e. 20 min before and 60 min after tetanization. As indicated by the slight leftward shift in the E-S relationship in Fig. 3A, LTP in tetanus only controls was characterized by a greater increase in the population spike amplitude than that predicted by the increase in EPSP slope. However, 5 Hz stimulation alone (Fig. 3B) caused a rightward shift in the E-S relationship, indicating a decrease in E-S coupling. No change in E-S coupling was seen in any of the groups that received a tetanus followed by 5 Hz stimulation, such as the 10 s group shown in Fig. 3C.

Linear regression lines were fitted to the scatter plots relating EPSP slope to population spike amplitude for each animal before and after tetanization (or before and after 5 Hz stimulation in the case of the 5 Hz only group). The spike amplitude elicited by a 75% maximal EPSP slope was interpolated from each regression equation. This value was compared before

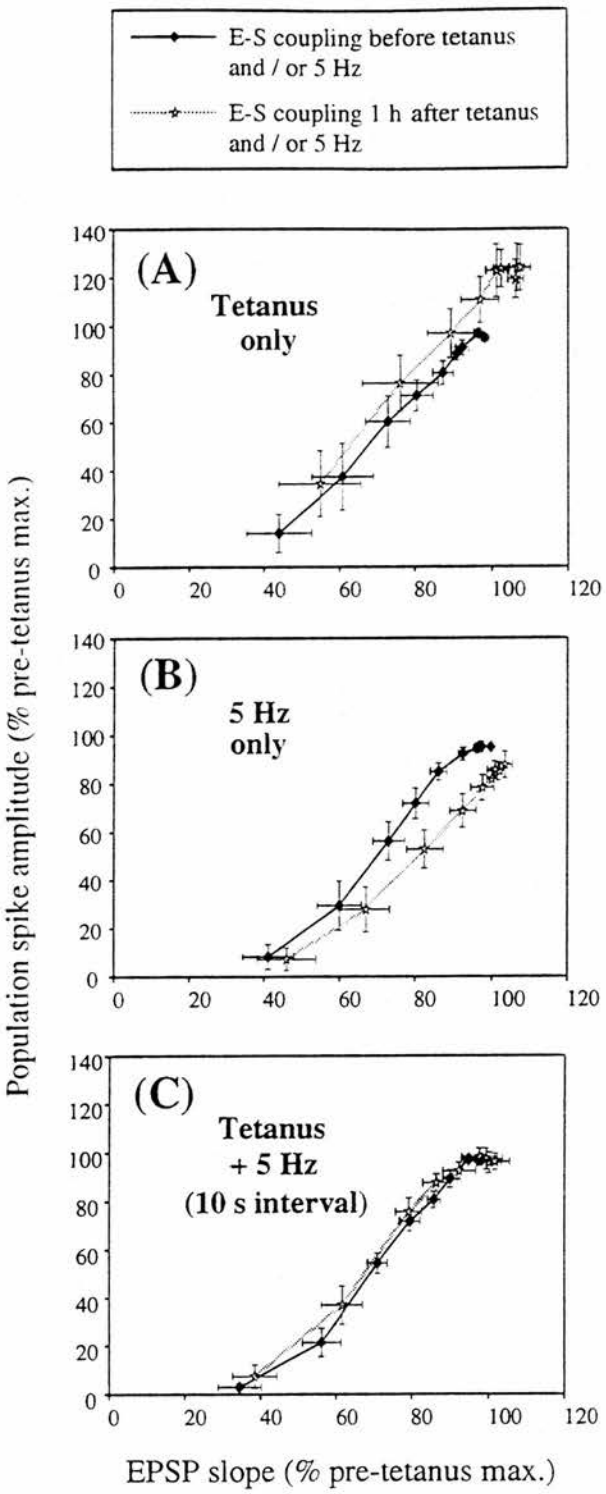


FIG. 3. Mean relationship between population spike amplitude and EPSP slope at each point on the I/O curve recorded before and after tetanization. (**A**) Tetanus only control group: the leftward shift indicates that tetanization induced a slight E-S potentiation. (**B**) 5 Hz only group: the rightward shift indicates that 5 Hz stimulation alone caused an E-S depression. (**C**) 10 s group: no change in E-S coupling was observed in animals receiving both a tetanus and 5 Hz stimulation, such as the 10 s group illustrated here.

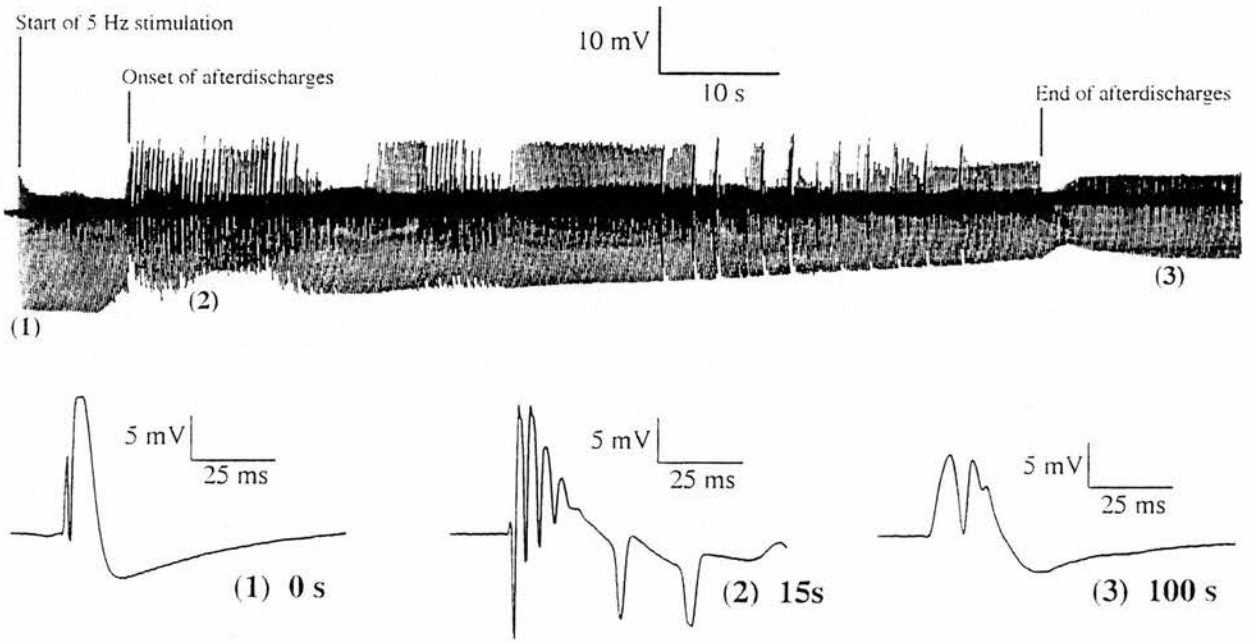
id after tetanization (or before and after 5 Hz imulation alone). A significant group by time point teraction was revealed ($F(6,38) = 3.96$; $p < 0.01$; ithin-subject ANOVA). Subsequent analysis of mple effects revealed significant E-S potentiation in ie tetanus only group ($F(1,38) = 6.89$; $p < 0.05$), and gnificant E-S depression in the 5 Hz only group $^2(1,38) = 11.90$; $p < 0.01$). No significant changes in -S coupling were obtained in any group receiving tetanus followed by 5 Hz stimulation (10 s: $F < 1$; 0 s: $F < 1$; 2 min: $F < 1$; 10 min: $F < 1$; 30 min: $(1,38) = 2.92$; $p > 0.05$).

pileptiform afterdischarges induced by 5 Hz stimuation: 5 Hz stimulation was always accompanied y epileptiform activity characterized by seizure-ke afterdischarges and multiple population spikes. igure 4 shows a continuous polygraph record uring 5 Hz stimulation. The large negative deflecions are positive-going EPSPs. In this example, afterischarges began within 10 s of the start of 5 Hz timulation and persisted for ~1.5 min, after which o further episodes of afterdischarge activity were bserved. Representative sample EPSPs at three ifferent time points are illustrated.

The afterdischarge onset latency and total duration vere measured for each rat (data not shown). Analysis of data from all rats revealed that these wo indices of the severity of afterdischarge activity were negatively correlated, i.e. the more rapidly afterdischarges began, the longer they persisted (correlation coefficient, $r = -0.41$; $p < 0.05$). However, no significant group difference was found in afterdischarge onset latency ($F(5,33) = 2.17$; $p > 0.05$) or duration ($F < 1$), indicating that seizure-like activity was equally severe regardless of the interval between tetanus and 5 Hz, or indeed regardless of whether a tetanus was delivered at all. Similarly, there was no overall correlation between afterdischarge onset latency or duration and the level of EPSP slope or population spike LTP obtained 1 h after tetanization (data not shown). These findings indicate that the time window during which LTP is vulnerable to reversal cannot be explained as an artifact of the severity of afterdischarges elicited by 5 Hz stimulation.

Failure of MCPG to block the reversal of LTP with 5 Hz stimulation: Consistent with our previous findings,[16] (*R,S*)-MCPG did not block LTP induced by tetanization 30 min after the end of infusion ($n = 3$; data not shown). The reversal of LTP with 5 Hz stimulation delivered 10 s after tetanization was also unaffected ($n = 3$; data not shown).

Failure of 1 Hz stimulation to reverse LTP: The delivery of a 10 min period of 1 Hz stimulation starting 10 s after tetanization ($n = 6$) had no effect



G. 4. Sample polygraph record during 5 Hz stimulation. The large negative deflections represent positive-going EPSPs. Epileptiform afterscharges began abruptly within 10 s of the start of 5 Hz stimulation, and continued for ~1.5 min before subsiding. Representative EPSPs corded at 0, 15 and 100 s after the start of 5 Hz stimulation (points 1, 2 and 3, respectively) are illustrated. The waveform recorded at 5 s displays the multiple population spikes characteristic of afterdischarge activity.

on either EPSP slope LTP or population spike LTP, relative to controls ($n = 6$) carried out at the same time (data not shown). Afterdischarges were never observed during 1 Hz stimulation.

Discussion

The present experiment reveals that dentate LTP *in vivo* exhibits a limited vulnerable phase, lasting less than 10 min after induction, during which potentiation can be disrupted by 5 Hz stimulation. Neither the induction of LTP, nor its reversal, was blocked by the application of the mGluR antagonist, MCPG. Despite the fact that tetanization alone resulted in E-S potentiation, and 5 Hz stimulation alone resulted in E-S depression, no changes in E-S coupling were seen in any group receiving a high frequency tetanus followed by 5 Hz stimulation. These results suggest that, in contrast to its time-dependent effects on LTP, 5 Hz stimulation causes a time-independent reversal of E-S potentiation, perhaps resulting from a non-specific depression of granule cell excitability.

The cellular mechanisms underlying the reversal of LTP in the present study remain unknown. It may be significant that stimulation at 5 Hz, a frequency within the range of the hippocampal theta rhythm, was effective in reversing LTP, whilst 1 Hz stimulation had no effect. The firing of neurons in area CA1 and the dentate gyrus of behaving rats is characterized either by high frequency bursts known as complex spikes, or by the firing of single action potentials.[17] Both forms of activity are frequently phase-locked to the theta rhythm. It has been suggested that the firing of complex spikes may represent a mechanism for the induction of a naturally occurring form of synaptic potentiation, whilst the firing of single pulses phase-locked to theta may serve to reverse such potentiation.[6] It has recently been reported that the exploration of a novel environment results in a complete reversal of CA1 LTP.[18] Exploration was accompanied by an increase in hippocampal EEG activity in the 6–8 Hz range, a result still obtained after controlling for the level of motor activity. Hence, hippocampal activity at theta frequencies may be involved in the experience-dependent erasure of synaptic enhancement, and as such may represent an active forgetting mechanism, working in concert with the storage of new information.

However, stimulation at theta frequencies readily induces seizure-like afterdischarges.[19,20] In the present study, activity of this kind was always observed during 5 Hz stimulation, but never during 1 Hz stimulation. As such, it is possible that LTP reversal by 5 Hz stimulation, rather than being a candidate mechanism for the active forgetting of information, may have more in common with various neuropathological events. One recent study reported that the delivery of 3 Hz stimulation starting 1 min after tetanization resulted in an apparent depotentiation of dentate LTP in awake rats, but only when accompanied by afterdischarge activity.[15] However, this depotentiation was found to be transient, and was interpreted as a non-specific depression of responses resulting from seizure generation. This possibility cannot explain the results of the present study, as afterdischarges were equally severe irrespective of the interval between tetanus and 5 Hz, yet no reversal of LTP could be induced 10 min or 30 min after tetanization.

It is possible that the time-dependent reversal of LTP by 5 Hz stimulation may be dependent on the activation of adenosine A_1 receptors. 5 Hz stimulation is known to result in an increase in adenosine release from hippocampal slices.[21] It has been reported that the application of adenosine A_1 receptor antagonists to CA1 slices prevents the induction of depotentiation,[6,22] and the reversal of LTP by excessive theta burst stimulation.[23] Furthermore, LTP can be reversed by the application of adenosine within 1 min, but not 5 min after tetanization.[24] This time window is similar to the period during which LTP was found to be vulnerable to reversal by 5 Hz stimulation in the present study. Interestingly, it may be significant that both adenosine A_1 receptor activation and low frequency stimulation are associated with a decrease in E-S coupling in area CA1 and the dentate gyrus, respectively.[25,26] These results are reminiscent of the E-S depression reported here after the delivery of 5 Hz stimulation without prior tetanization.

The physiological relevance of long trains of low frequency stimulation such as a 10 min period of 5 Hz is questionable. The possibility that LTP induced using a more physiologically realistic tetanus might be reversed by a brief period of theta stimulation remains a topic for future experiments. Nevertheless, regardless of the physiological significance of the present findings, the ability to preferentially erase recently induced LTP, whilst sparing established LTP, might provide a novel alternative to pharmacological intervention in future behavioural studies of synaptic plasticity and learning.

Conclusion

These results reveal that a 10 min train of 5 Hz stimulation reverses dentate LTP *in vivo* only when delivered up to 2 min after tetanization. This finding is reminiscent of the effects of adenosine A_1 receptor activation.[24] However, the mechanism and physiological significance of LTP reversal by 5 Hz

imulation are currently unknown. Despite this, the ility to preferentially erase recently induced LTP ight provide a useful tool for behavioural studies the relationship between LTP and learning.

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